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POLYMORPHISM AND TRANS FATTY ACID DISTRIBUTION IN MILKFAT

BY

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A THESIS
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Polymorphism and Trans Fatty Acid Distribution in Milkfat", submitted by Irwin Lewis Woodrow, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The distribution of trans unsaturated fatty acids among and between milkfat glycerides was investigated with infra-red spectroscopy and pancreatic lipase hydrolysis. The trans fatty acids were found to be esterified mainly in the 1- and 3- positions of the glycerides and the distribution among glycerides followed a pattern of about equal distribution in glycerides groups divided on the basis of melting point by fractional crystallization from acetone. This distribution was in contrast to the cis unsaturated fatty acid which were absent in the highest melting fraction and increased in amount with decreasing melting point of fractions. The fatty acids, lauric, myristic, group A and palmitic were found to be incorporated preferentially in the 2-position while the fatty acids, stearic and oleic were esterified in the 1- and 3- positions.

Polymorphism was studied by infra-red spectroscopy and X-ray diffraction. All three polymorphic forms, alpha, beta-prime and beta were found in slowly cooled milkfat and rapidly cooled milkfat from 40°C to 0°C. The polymorphic forms observed in the high melting fraction depended on the thermal treatments. The beta form was obtained by fractional crystallization from acetone, the beta-prime form by slow cooling the melt and the alpha form by rapidly cooling the melt. A broad and diffuse X-ray diffraction pattern was obtained with milkfat at room temperature. Removal of most of the liquid oil by pressure filtration improved the resolution of the X-ray diffraction pattern.

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I wish to dedicate this thesis to my Mother, Father, my brothers Gary and Adrian, my sister, Beryl-Ann and also to my roommate Al Purkess.

TABLE OF CONTENTS

Page Number

INTRODUCTION.. 1

LITERATURE REVIEW..................................... 3

Fatty Acid Composition of Milkfat..................... 3

Trans Acids in Milkfat.............................. 3

Trans Fatty Acid Determination...................... 8

Infra-red Spectroscopy................................ 8

Gas-Liquid Chromatography............................. 11

Other Methods... 12

Distribution of Fatty Acids in the Glycerides of Milkfat 12

Polymorphism.. 14

General... 14

Natural Fats.. 19

Milkfat... 22

EXPERIMENTAL METHODS.................................. 27

Distribution of *Trans* Fatty Acids in Milkfat........ 27

Source of Milkfat..................................... 27

Interesterification................................... 27

Fractional Crystallization from Acetone............... 27

Pancreatic Lipase Hydrolysis.......................... 28

Extraction of Lipids from Hydrolyzate................. 29

Separation of Glycerides by Column Chromatography 31

Charaterization of Material Remaining in Column 31

Charaterization of Lipid Classes by Thin Layer Chromatography.. 33

TABLE OF CONTENTS (CONTINUED)

Page Number

Determination of Fatty Acid Composition.......... 33

Determination of *Trans* Isomer Content in Original Samples.. 34

Determination of Percentage *Trans* as Trielaidin in 2-Monoglycerides from Hydrolyzate................ 35

Determination of Polymorphic Forms of Milkfat........... 37

Infra-red Spectroscopy.......................... 37

X-ray Diffraction............................... 40

RESULTS.. 42

Trans Fatty Acid Distribution in Milkfat, Interesterified Milkfat and their Fractions........................... 42

Fractional Crystallization...................... 42

Saponification Values of Milkfat, Interesterified Milkfat and their Fractions...................... 42

Chromatographic Separation of Partial Glycerides in Hydrolyzate................................ 43

Characterization of Material Remaining in Column. 44

Purity Check of Partial Glycerides by Thin Layer Chromatography................................ 44

Percentage *Trans* Fatty Acids in Milkfat, Interesterified Milkfat and their Fractions.......... 45

Trans Determination in 2-Monoglycerides.......... 45

Fatty Acid Composition of 2-Monoglycerides....... 46

Polymorphism.. 48

Milkfat.. 48

High Melting Fraction of Milkfat................ 49

TABLE OF CONTENTS (CONTINUED)

Page Number

DISCUSSION.. 77

Trans Distribution in Milkfat.......................... 77

Fatty Acid Distribution in Milkfat....................... 80

Characterization of Residue Remaining in Column after Elution of Partial Glycerides......................... 81

Polymorphism in Milkfat.................................. 82

BIBLIOGRAPHY.. 87

LIST OF TABLES

Table Number		Page Number
I	Fatty acid composition of milkfat from Boudreau & deMan, 1965a	5
II	Percentage of *trans* unsaturated fatty acids in summer and winter milkfat as reported in the literature	7
III	Infra-red absorption maxima of unsaturated systems from O'Connor, 1956	10
IV	Percentage of various fatty acids located at the 2-position of milkfat glycerides as reported in the literature	15
V	Comparison of Lutton's and Malkin's data and nomenclature of the polymorphic forms for tristearin.	18
VI	Fatty acid composition of milkfat, its fractions obtained from fractional crystallization from acetone and the 2-monoglycerides obtained by hydrolysis	51
VII	Fatty acid composition of interesterified milkfat, its fractions obtained from fractional crystallization from acetone and the 2-monoglycerides obtained by hydrolysis	52
VIII	Saponification values of milkfat, interesterified milkfat and their fractions calculated from the average molecular weight of the fatty acids	53
IX	Percentage monoglycerides recovered from the hydrolysis of milkfat, interesterified milkfat and their fractions	54
X	Percentage *trans* and unsaturated fatty acids and their ratio in milkfat, interesterified milkfat, and their fractions	55
XI	Percentage fatty acids esterified in 2-position of milkfat and its fractions	56
XII	Percentage fatty acids esterified in 2-position of interesterified milkfat and its fractions	57

LIST OF TABLES (CONTINUED)

Table Number		Page Number
XIII	Fatty acids that exceed 10% difference in the percentage fatty acids in the 2-position of milkfat, interesterified milkfat and their fractions.	58
XIV	Infra-red absorption peaks and short spacings from X-ray diffraction obtained with milkfat and high melting fraction, either slowly or rapidly cooled and the high melting fraction crystallized from acetone...................................	59
XV	Polymorphic literature data from infra-red spectra and short spacings from X-ray diffraction.........	83

LIST OF FIGURES

Figure Number		Page Number
I	Transformation of polymorphs of different common fats under different tempering and storage treatments.	21
II	X-ray diffraction patterns of milkfat covering the range 2θ angles from 2 to 34° (A) slowly cooled, (B) rapidly cooled. From deMan (1961a).	24
III	pH controller.	30
IV	Column chromatography setup.	32
V	Determination of percentage trans unsaturation from the height of the peak at 10.36 μ.	36
VI	Perkin Elmer 21 infra-red spectrophotometer.	39
VII	Infra-red spectrum of residue remaining in the column after separation of mono, di and triglycerides.	60
VIII	Thin layer chromatogram of milkfat, hydrolyzed milkfat and lipid classes separated by column chromatography.	61
IX	Infra-red spectra of the 2-monoglycerides obtained after pancreatic lipase hydrolysis of the -15°C fraction of milkfat.	62
X	Infra-red spectra of the 2-monoglycerides obtained after pancreatic lipase hydrolysis of trielaidin.	63
XI	Gas chromatograms of methyl esters of +15°C milkfat fraction (A) and the methyl esters of the 2-monoglycerides of the +15°C milkfat fraction after hydrolysis (B)	64
XII	Infra-red spectra of milkfat at 40°C, (A) after rapid cooling to 0°C (B), and with subsequent holding at 5°C for 10 hours (C)	65
XIII	Infra-red spectra of milkfat determined during cooling of the fat from 40-0°C at 4°C intervals.	66
XIV	X-ray diffraction pattern of slowly cooled milkfat after cooling slowly from 40-0°C.	67

LIST OF FIGURES (CONTINUED)

Figure Number — Page Number

XV X-ray diffraction pattern of rapidly cooled milkfat after cooling rapidly from 40-0°C by dry ice....... 68

XVI Infra-red spectra of high melting glyceride fraction (+15°C acetone crystallization) at 65°C (A) and after rapid cooling to 10°C (B).................... 69

XVII X-ray diffraction pattern of high melting fraction cooled rapidly from 60°C to 10°C and run at room temperature..................................... 70

XVIII Infra-red spectra of high melting fraction cooled slowly from 60°C to 10°C at 4°C per hour........... 71

XIX X-ray diffraction pattern of slowly cooled high melting fraction from 65°C to 10°C.................. 72

XX Infra-red spectrum of high melting fraction from acetone.. 73

XXI X-ray diffraction pattern of high melting fraction from acetone....................................... 74

XXII X-ray diffraction pattern of milkfat crystallized at room temperature (25°C). Determination at 25°C.. 75

XXIII X-ray diffraction of the solid portion of milkfat crystallized at room temperature and separated from the liquid by pressure filtration. Determination at 25°C... 76

INTRODUCTION

The complexity of milkfat is a direct result of the wide variety of fatty acids present. The fatty acids range from tetranoic to eicosahexanoic, many of these also occurring with one or more double bonds, and in addition there are several iso and anteiso acids (Garton, 1963). The unsaturated fatty acids occur in the trans and cis configuration. The trans fatty acids are produced in the rumen by microbial hydrogenation of the polyunsaturated lipids of the feed. The trans fatty acids can be used as natural tracers for the determination of some aspects of the biosynthesis of milkfat. In this thesis the trans fatty acid content and distribution among and within the glycerides of milkfat were determined.

The distribution of fatty acids in the glyceride molecule has a considerable effect on the physical properties of milkfat. The melting range of the glycerides of milkfat is between -40°C and +40°C. Thus at any temperature in this range there is present a certain amount of liquid fat which acts as a solvent for some of the solid glycerides. The polymorphic behavior of milkfat and the high melting fraction obtained by fractional crystallization from acetone at +15°C is influenced by the wide melting range. This can be observed by studying the crystal modifications of milkfat and its high melting fraction.

The technique of infra-red spectroscopy has recently been introduced to the study of polymorphism in fats (Chapman, 1956). Infra-red spectroscopy is also widely used for the determination of trans content in lipids. The techniques used in this work, in addition to infra-red spectroscopy and

X-ray diffraction included gas-liquid chromatography, column chromatography, thin layer chromatography and pancreatic lipase hydrolysis.

REVIEW OF LITERATURE

FATTY ACID COMPOSITION OF MILKFAT

Boatman, Decoteau & Hammond (1961) reported the presence of at least 60 different fatty acids in milkfat. The proportions of these fatty acids present depend on a number of factors, including inherited variations, seasonal variations, temperature, age of cow, stage of lactation and feed. The influence of these factors on fatty acid composition has been discussed by Hilditch & Williams (1964). Milkfat contains fatty acids with chain length of 4 to 26 carbon atoms. Magidman *et al*. (1962) classified the fatty acids in milkfat in the following categories.

Saturated fatty acids:

(1) Straight-chain even-numbered fatty acids
(2) Straight-chain odd-numbered fatty acids
(3) Branched-chain, alicyclic and keto fatty acids

Unsaturated fatty acids:

(1) Even-numbered monounsaturated fatty acids
(2) Odd-numbered monounsaturated fatty acids
(3) Polyunsaturated fatty acids

The concentrations of the major fatty acids in milkfat are listed in Table I (Boudreau & deMan, 1965a).

Trans Acids in Milkfat

The listing of fatty acids in tabular form usually takes place according to carbon chain length and unsaturation. In natural fats containing unsaturated fatty acids the *cis* form is usually predominant, however, *trans*

acids may also be present. Trans fatty acids are more stable and have higher melting points than the corresponding cis acids. Because fatty acids with trans configuration have higher melting points their physical properties resemble the saturated fatty acids more than the unsaturated ones.

The unsaturated fatty acids of milkfat have carbon chain lengths greater than ten. Hilditch & Longenecher (1938) reported the presence of 0.4% decenoic, 0.9% dodecenoic, 1% tetradecenoic and 3.7% hexadecenoic acids with ethenoid linkages at carbon 9 position with respect to the carboxyl group. The geometrical configuration of these fatty acids was cis. The major unsaturated fatty acid in milkfat is octadecenoic acid which consists of 27% of the total fatty acids. Odd numbered monoene fatty acids have also been found in milkfat in trace amounts. Hanson, Shorland & Cooke (1960) isolated cis 9-heptadecenoic acid from milkfat in the amount of 0.06% of the total fatty acids.

Other unsaturated fatty acids in milkfat include the C-18 polyethenoic acids. Green & Hilditch (1935) suggested that the octadecadienoic acids of milkfat are principally composed of geometric (cis,trans and trans,cis) isomers of linoleic acids. Shorland (1950) was able to determine the different linolenic acids in milkfat. deJong & van der Wel (1964) identified some isolinoleic acids occurring in butterfat by using thin layer chromatography on silica gel impregnated with silver nitrate. Because of the divergent reports on the nature of the polyethenoic fatty acids with 18 carbons in butterfat the matter was investigated by Sambasivarao & Brown (1962). They used bromination in their studies and found that C-18 dienoic acids exist mostly in the linoleic or cis,cis, and cis, trans configurations. Garton (1963) re-

Table I. Fatty Acid Composition of Milkfat (Boudreau & deMan, 1965a)

Fatty Acid[a]	Percentage[b]
4:0	2.5
6:0	1.5
8:0	1.0
10:0	2.1
10:1	0.3
12:0	2.8
14:0	10.1
15:0 & 16:0^{iso}	2.1
16:0	26.7
16:1	3.0
17:0 & 17:0^{iso}	1.1
18:0	12.6
18:1	26.0
18:2	1.7
18:3	2.8

a. Number of carton atoms : number of double bonds

b. By weight

viewed the types of unsaturated fatty acids in milkfat.

Bertram (1928) discovered the existence of vaccenic acid (trans-11-octadecenoic) in milkfat. Subsequent studies indicated levels of vaccenic acid in milkfat of 1.4-4.7% (Grossfeld & Simmer, 1930), 1.8-3.9% (Brouwer & Jonker-Scheffener, 1946) and 0.3-3.5% (Kuzdzal-Savoie, 1964).

Besides vaccenic acid other trans fatty acids exist in milkfat (Hilditch & Williams, 1964). Smith, Freeman & Jack (1954) gave evidence of trans isomers of fatty acids with carbon numbers of 12, 14, 16 and 18. The trans octadecenoic acids also exist in positional isomers. By oxidation studies and paper chromatography Phatak & Patwardhan (1953) identified the trans C-18 monoethenoid acids with double bonds at carbon 8, 9, 10, 11 and 12. Trans octadec-16-enoic acid was characterized in milkfat by Backderf & Brown (1958) and Hansen & Cooke (1961). The experimental proof was obtained by the presence of thapsic acid (hexadecanedioic acid) as one of the products of oxidation of milkfat.

The percentage of the trans fatty acids in milkfat varies with season. Hartman, Shorland & MacDonald (1955) observed that the trans acids arise mainly from hydrogenation of dietary unsaturated acids by bacteria in the rumen. The trans fatty acid content in summer is about twice as high as the amount found in winter. Trans contents found by several workers in winter and summer milkfat are given in Table II.

Table II. Percentage of trans unsaturated fatty acids in summer and winter milkfat as reported in the literature.

Reference	% Trans Winter	% Trans Summer
Cornwell *et al.* (1953)	3.5	8
Patton *et al.* (1960)	2.5	6.4
deMan (1961)	4.9	6.6
Kaufmann, Volbert, & Mankel (1961)	2	9
Antila (1965)	1.4	7.2
Galoppini & Lotti (1965)	8.1	9.6
Kuzdzal-Savoie & Raymond (1965)	2.2	7.0

TRANS FATTY ACID DETERMINATION

Infra-red Spectroscopy

Infra-red spectroscopy has been used extensively for the determination of *trans* unsaturated acids (O'Connor, 1961). The infra-red absorption bands arise as a result of changes in vibrational energy levels of a molecule. A specific absorption is associated with some vibrating part of the molecule, and influenced by the "molecular environment" of the molecule as a whole. The infra-red absorption bands are assigned to vibrations of parts of molecules which are called "functional groups." Rasmussen, Brattain & Zucco (1947) observed that the *trans* group in unsaturated compounds exhibits a strong band at 10.3 μ (970 cm^{-1}). This band has been shown to arise from a C-H deformation about a *trans* double bond in long chain hydrocarbons (Sheppard & Sutherland, 1949). This phenomenon was used for the development of a quantitative method for determination of *trans* unsaturated fatty acids or their derivatives (Shreve *et al.*, 1950). Swern *et al.* (1950) and Jackson & Callen (1951) compared the infra-red method with that of Twitchell (lead salt alcohol method) for the determination of *trans* octadecenoic acids and esters. They found that the infra-red method was superior. The reason for the failure of the Twitchell method apparently lies in the increasing solubility of lead isooleate with increase in the concentration of *trans* isooleic acid.

Jackson *et al.* (1952) studied the isomers of linoleic acids, and observed that the bond characteristics of isolated *trans* double bonds are approximately additive. This was proven by the fact that linolelaidate has

about twice the absorption of elaidate and *cis-trans* linoleate has about the same absorption as elaidate. They could also show that various conjugated combinations give rise to bond maxima at specific wavelengths (Table III). Ahlers *et al.* (1953) confirmed these correlations and suggested that the positions of the maxima in the spectra of conjugated acids containing *trans* bonds involve a hypsochromic shift from the position of the isolated *trans* bond, this shift is small but significant and consistent with the effect of conjugation and the C-H deformation frequency of the system.

Chipault & Hawkins (1959) developed a method for the quantitative determination of conjugated methyl *cis-trans* and *trans-trans* octadecadienoates by infra-red spectroscopy.

The American Oil Chemists' Society proposed a Tentative Method (Cd 14-61), for the determination of isolated *trans* isomers by infra-red spectroscopy. In this method a baseline technique is used to obtain quantitative results, and can be applied to triglycerides, methyl esters and fatty acids.

On the basis of a collaborative study by the Spectroscopy Committee of the American Oil Chemists' Society (1960) on the determination of *trans* isomers in fatty acids it was concluded that if the *trans* content is lower than 15 per cent an excessive background correction results from the proximity of the strong carboxyl absorption band. It was recommended that the fatty acids be converted to their methyl esters if the *trans* content is lower than 15 per cent. Firestone & La Bouliere (1965) studied the American Oil Chemists' Society method and observed that when triglycerides were used for the deter-

Table III. Infra-red absorption maxima of unsaturated systems (O'Connor, 1956).

Unsaturated System	Wavelength Maxima (microns)
isolated *trans*	10.34
cis,trans conjugated	10.17
trans,trans conjugated	10.12
cis,cis,trans conjugated	10.11
cis,trans,trans conjugated	10.09
trans,trans,trans conjugated	10.06

mination the trans values were 2-3 per cent high, whereas when the triglycerides were converted to their methyl esters their trans content was 1.5-3 per cent low. They recommended that corrections should be made when calculating trans content of triglycerides, or their methyl esters if the concentration is low.

Szonyi, Tait & Crashe (1962) used a differential infra-red spectrophotometer method for determining trans unsaturation in triglycerides. They found the method applicable to samples with low trans concentration.

Infra-red spectroscopy can also be applied to other fields of lipid research which include polymorphism, autooxidation and rancidity studies and polymerization. Reviews of the applications of infra-red spectroscopy to lipid research including trans isomers have been compiled by O'Connor (1955, 1961), Chouteau (1961), Kaufmann, Volbert & Mankell (1959), Kauffman (1964) and Chapman (1965).

Gas-Liquid Chromatography

The separation of cis and trans fatty acids was first reported by James & Martin (1956) for methyl esters of unsaturated fatty acids. Using an Apiezon N column the unsaturated acids with cis-configuration were eluted faster than those with trans configuration. A capillary column with high efficiency and a sensitive ionization detector must be used to obtain cis, trans separation.

Lipsky, Landowne & Lovelock (1959) used high efficiency columns with theoretical plate efficiency approaching 1000 theoretical plates per foot,

which separated elaidate from oleate but not linoleate from linolenate.

Litchfield, Reiser & Isbell (1963) reviewed the analysis of cis, trans fatty acid isomers by gas-liquid chromatography. They presented data on the resolution of linolenate geometric isomers on both polyester and Apiezon columns. Nitrile silicone columns have also been used successively for the separation of geometrical isomers (Litchfield et al., 1964).

Other Methods

The ability of silver ions to form weak complexes with olefins and the difference in stability of silver ion complexes of cis and trans isomers enables the separation of cis and trans compounds (Lucas, Moore & Pressman, 1943). By applying this principle, quantitative separation of cis and trans isomers can be accomplished. The techniques that have been used include counter current distribution (Dutton, Scholfield & Jones, 1961; Scholfield et al. 1963; Scholfield Butterfield & Dutton, 1966), macroreticular exchange resin (Emken, Scholfield & Dutton, 1964), column chromatography (de Vries, 1962; 1963), and thin layer chromatography (Blank, Verdino & Privett, 1965).

DISTRIBUTION OF FATTY ACIDS IN THE GLYCERIDES OF MILKFAT

The determination of glyceride structure of milkfat is a difficult task. Lovern (1955) reports that if a fat contains 'n' fatty acids then the number of possible glycerides would be $(n^3 + 3n^2 + 2n) \div 6$. Thus if milkfat contains 60 fatty acids (Boatman, Decoteau & Hammond, 1961) there would be 37,820 possible glycerides. This number does not include the positional or

stereo isomers.

Several methods have been developed to simplify the problem by preliminary separation. The method most commonly used is that of fractional crystallization (Hilditch & Williams, 1964). These fractions can be obtained either by fractionation from a solvent such as acetone (Chen & deMan, 1966) or in absence of solvent (Moore, Richardson & Amundson, 1965). The fractions are separated from the liquor by filtration or centrifugation (Rolland & Riel, 1966). Youngs & Sallans (1958) pointed out that crystallization of triglycerides from acetone is dependent on saturation rather than unsaturation of the individual fatty acids. The high melting fractions obtained have low content of short chain and unsaturated fatty acids while the low melting fraction have low content of stearic and higher contents of short chain fatty acids.

Oxidation procedures (Youngs, 1961; Kartha, 1953) and use of mercuric acetate adducts (Kerkoven & deMan, 1966) enable the separation of saturated from unsaturated glycerides. Fractionation of milkfat has also been accomplished by utilizing counter current distribution (Smith, Freeman & Jack, 1965; Haab, Smith & Jack, 1959). With thin layer chromatography it is possible to separate the short chain triglycerides from the longer chain triglycerides in milkfat (Blank & Privett, 1964). Kuksis, McCarthy & Beveridge (1963) used gas-liquid chromatography to separate the triglycerides of milkfat on the basis of carbon number or molecular weight. With the preceding methods it is not possible to determine the fatty acid distribution in the glyceride molecules. This can be accomplished by use of the pancreatic

lipase technique. Pancreatic lipase (steapsin) specifically hydrolyzes the fatty acids esterified on the external positions of the glycerides (Mattson & Volpenhein, 1961). Thus this method can be used to determine the positioning of fatty acids in the glyceride molecule. Pancreatic lipase besides having a specificity for 1 and 3 positions of the glycerides also has a rate specificity for short chain fatty acids with chain length less than eight carbon atoms. Thus hydrolysis can occur also in the 2-position if a short chain fatty acid is occupying that position. Because of this specificity a greater proportion of short chain fatty acids will be found as free fatty acids after hydrolysis (Entressangles *et al.*, 1961; Clement *et al.*, 1962; Boudreau & deMan, 1965). Table IV lists the proportions of fatty acids found in 2-monoglycerides of milkfat after hydrolysis.

POLYMORPHISM

General

Polymorphism describes the phenomenon of the existence of two or more crystalline modifications of the same substance. To understand the complexity of this subject one must realize that milkfat consists of a multitude of glycerides which by themselves may exist in different polymorphic forms.

Reviews of the polymorphic forms of glycerides have been published by Ferguson & Lutton (1941); Lutton (1950), (1955); Chapman (1962), (1965a); Malkin (1954) and Larsson (1964). Different methods have been used to determine the existence of polymorphic forms. These include melting point, cooling and heating curves, microscopy, dilatometry, dielectric studies,

Table IV. Percentage of various fatty acids located at the 2-position of milkfat glycerides as reported in the literature.

Fatty Acid	Literature				
	McCarthy, Patton & Evans (1960)	Ast & Vander Wal (1961)	Jensen, Sampugna & Pereira (1964)	Boudreau & deMan (1965)	Sampugna *et al.* (1966)
4:0	-	-	-	0.2	2.1
6:0	0.1	0.7	0.9	0.3	1.8
8:0	1.1	0.6	3.1	1.0	3.1
10:0	4.7	2.6	6.8	2.8	6.7
10:1	-	0.4	0.6	0.3	0.4
12:0	5.4	2.7	7.8	3.9	7.6
12:1	-	0.2	0.7	-	0.8
14:0	20.0	13.8	22.2	19.5	21.5
A[a]	-	5.1	2.9	5.2	1.6
16:0	40.0	28.2	33.2	32.7	30.8
B[b]	2.3	4.6	2.8	5.3	4.2
18:0	8.7	9.6	7.3	4.2	4.3
18:1	15.0	24.1	10.8	20.5	13.7
18:2	1.9	3.3	0.9	2.5	1.4
18:3	-	1.8	-	0.9	tr.

a. A includes fatty acids 14:1, 15:0, $16:0^{iso}$

b. B includes fatty acids 16:1, 17:0, $17:0^{iso}$

X-ray diffraction, infra-red spectroscopy, nuclear magnetic resonance, electron diffraction and mechanical resonance dispersion. The most widely used methods are thoseof X-ray diffraction, and more recently infra-red spectroscopy.

The determination of the crystalline order of the atoms or molecules can be derived from the X-ray diffraction patterns. This is because the molecules in the crystal behave as a three dimensional diffraction grating. The arrangement of layers in these so called gratings is parallel to one another and the perpendicular distance between the successive planes can be determined directly by X-ray powder diffractometry. The short and long spacings observed are related to the different crystal forms (Hoerr, 1964; Gunstone, 1964).

In infra-red spectroscopy, the group vibration that will distinguish between the different crystalline forms is the CH_2 rocking mode which absorbs in the 720 cm $^{-1}$ region. Davison & Corish (1955) emphasized that this band is associated with an essentially planar trans - configuration. Group vibrations of polymethylene chains couple to produce the vibrations of the complete polymethylene chain which are part of the glyceride molecule.

There has been a great deal of controversy over the different crystalline forms that can be found in individual glycerides. This controversy was mainly between the Malkin and Lutton groups. The main difference between these two groups stated by Lutton (1945) was that Malkin's & Meara's (1939) nomenclature arises from a faulty association of diffraction

patterns and melting points. The differences of these two groups are shown in Table V. In this thesis, reference will be made only to Lutton's nomenclature. It was found that there are three different polymorphic forms that can be detected in certain glycerides. These forms are called alpha, beta-prime and beta. The stability and melting point increase in this order. Lutton (1945) and Knoop & Samhammer (1961) observed the three forms when studying polymorphism of tristearin to trilaurin homologs. The mixed glycerides of palmitic and stearic acids show remarkable individuality (Lutton, Jackson & Quimby, 1948). Besides the alpha form, 2-palmitodistearin exhibits only the beta modification, whereas 2-stearyldipalmitin occurs only in the beta-prime form. The other mixed triglycerides exhibit all three forms. Lutton (1966) found that the symmetrical compounds of palmitoyl and stearoyl diolein exhibit alpha, beta-prime-2, and stable beta-3 forms where -2 implies a long spacing of double chain length and -3 for the long spacing corresponding to triple chain length. The unsymmetrical compounds show alpha (presumed) and stable beta-prime-3 form. It is evident from the results of Lutton that there is no law or order of prediction of the different polymorphic forms of mixed triglycerides.

Larsson (1964a, b, c, 1966) studied the crystal structure of the beta form of trilaurin, 2-monolaurin and the $beta_1$ and $beta_2$ form of racemic 1-monoglycerides. $Beta_1$ and $beta_2$ forms are closely related to the beta structure. The difference between $beta_1$ and $beta_2$ are their direction of chain tilt, $beta_1$ alternates in successive double layers, whereas, $beta_2$ alternates in successive single layers. In an earlier paper Larsson (1963)

Table V. Comparison of Lutton's and Malkin's data and nomenclature of the polymorphic forms for tristearin

Lutton

Melting Point	Short Spacings	Long Spacings	Name of Form
54°C	4.14 Å	50.6 Å	alpha
64	3.78, 4.18	46.8	beta-prime[a]
73.1	3.68, 3.84 4.61, 5.24	45.15	beta

Malkin

Melting Point	Short Spacings	Long Spacings	Name of Form
54.5°C	4.2 Å diffuse	....	gamma or glassy
65.0	4.2	50.6	alpha
71.5	3.7, 3.9 4.6, 5.3	45	beta

a. Beta-prime for mixed glycerides according to Malkin approx. 3.8, 4.2. The situation is analogous for other homologs.

examined the crystal structures of diglycerides. The crystal parameters and structural factors of the specific glycerides were worked out. Jensen & Mabis (1963) calculated the unit cell parameters of beta-tricaprin. The unit cells of the polymorphic forms were found to be hexagonal in the alpha form, orthorhombic in the beta-prime and triclinic or monoclinic in the beta form.

Chapman (1955) used infra-red spectroscopy to observe the different polymorphic forms of 1-monostearin, and showed that different forms give different spectra. From this preliminary study he extended his work to a variety of glycerides, including 1-monoglycerides, 1:3 diglycerides, 1:2 diglycerides, saturated triglycerides and mixed glycerides (Chapman 1956, 1956a, 1957, 1958, 1958a). A review of these papers was compiled by Chapman (1960).

The different polymorphic forms were characterized chiefly by the appearance and position of the CH_2 rocking mode which is located in the 720 cm^{-1} region of the infra-red spectrum. A doublet near 727 cm^{-1} and 719 cm^{-1} indicates that the hydrocarbon chains are in the orthorhombic or beta-prime form. A single band at 720 cm^{-1} indicates the presence of the beta form, while a single band at 717 cm^{-1} was attributed to the unstable alpha form.

Natural Fats

Polymorphism is important in products in which fats are the main constituent. Certain products require a specific polymorphic form to impart

the desirable characteristics, e.g. margarine, shortening and chocolate. Polymorphism will effect texture and appearance (Singleton, 1955; Haighton, 1963; Hannewijk, 1964; Sumi & Abe, 1965).

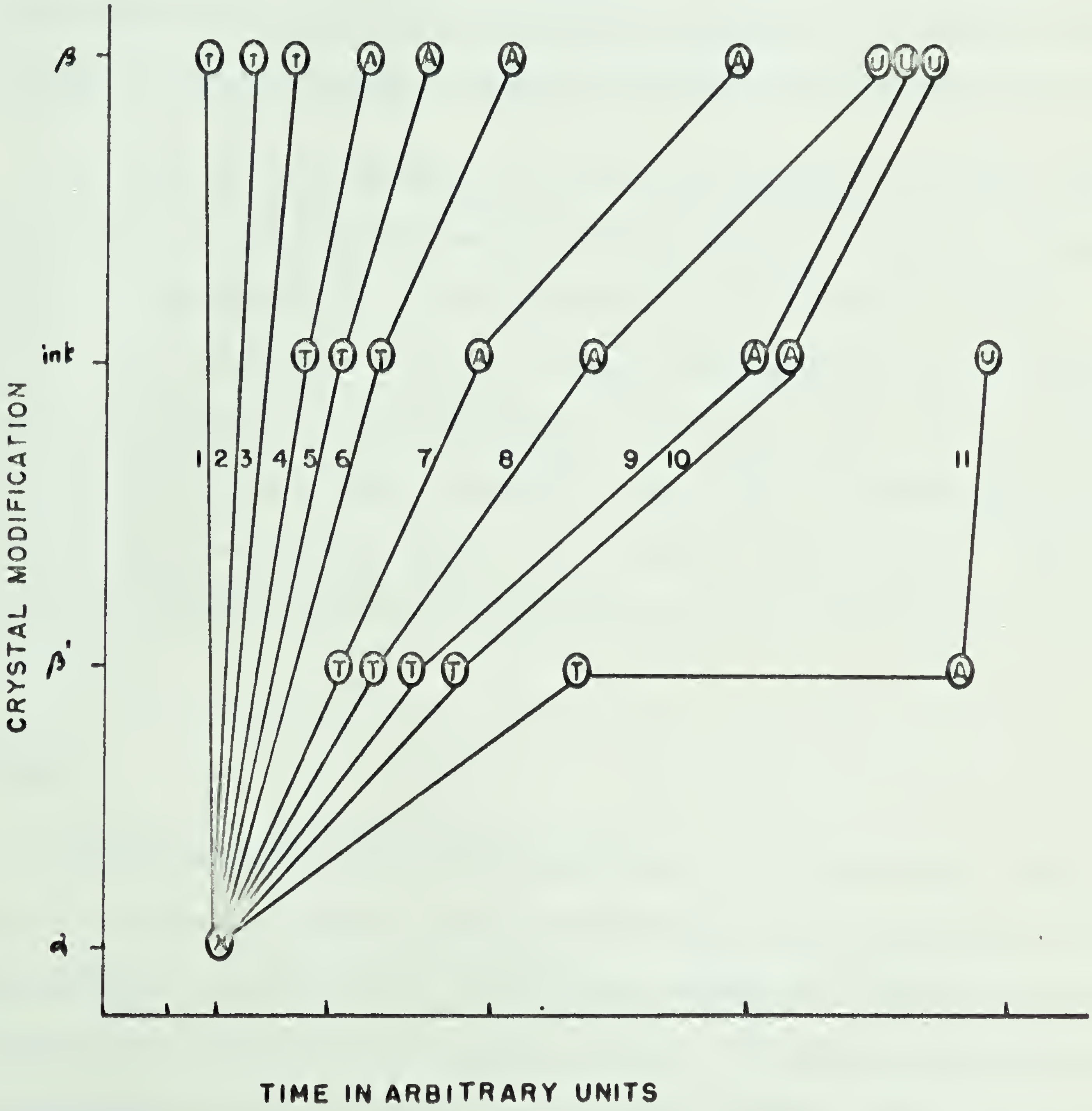
Merker, Brown & Wiedermann (1958) studied the transformation in texture of margarine formulated with different proportions of hydrogenated soybean and cottonseed oils with respect to their polymorphism. The graininess of the samples were related to the formation of the beta polymorph. The increase in the percentage of cottonseed oil increased the tendency for beta-prime formation which results in a smoother texture. These texture changes were related to crystal growth rather than polymorphic transformation. Crystal growth will depend on the amount of solids, rate of cooling and tempering temperature and directly contributes to the type of polymorphic form that is present. Crystal growth apparently is a factor in the changes in hardness of confectionary fats on storage (Feuge & Guice, 1959). Fats containing a sizable proportion of a liquid component will become softer on prolonged storage because the presence of the liquid component makes possible a gradual increase in crystal size.

Hoerr (1960) found that a wide variety of fats exhibit four polymorphic forms. The forms were designated alpha, beta-prime, intermediate and beta. The alpha, beta-prime and beta forms were identical to Lutton's while the intermediate did not vary considerably from Lutton's beta-prime-3 and beta-3. The 'intermediate' form exhibited strong short spacings at 4.62Å, 4.15Å and 3.75Å and a weak short spacing at 3.60 Å. Figure I illustrates the transformation of polymorphs of different common fats under dif-

Figure I. Relative rates of transformation of fats.

Letters in the circles refer to the following: X-form during crystallization. T-form after normal tempering. A-form after aging during normal storage. U-form after storage under unusual conditions.

Numbers on the curves identify the following fats: 1-Lard flakes. 2-Soybean flakes. 3-Cocoa butter. 4-Killing lard. 5-Partially hydrogenated lard. 6-Ordinary lard. 7-High I.V. lard. 8-Rearranged-lard flakes. 9-Cutting lard, partially hydrogenated soybean oil (low I.V.). 10-Tallow flakes, cottonseed flakes. 11-Rearranged lard, ordinary tallow, partially hydrogenated soybean oil (high I.V.), partially hydrogenated cottonseed oil.



ferent tempering and storage treatments. The fats with relatively simple glyceride composition transformed rapidly to the beta form, whereas those fats that contain a variety of mixed glycerides remain in the lower forms.

Hellman, Zobel & Senti (1955) noted that the solubility of monoglycerides in oil depends on their crystallinity. They examined the X-ray diffraction pattern of a 10% monoglyceride suspension. A prominent short spacing of 4.55 Å indicated that the beta crystal form occurred in the sample.

Cacao butter is considered to be one of the simplest of natural fats and consists of 19% 2-oleodistearin; 52% oleopalmitostearin; 9% palmitodiolein; 6% oleodipalmitin; and 2% palmitostearins (Hilditch & Williams, 1964). From polymorphic data the structural configuration of oleopalmitostearin was changed to palmitooleostearin, since there was a closer relationship of the X-ray diffraction pattern of cacao butter to palmitooleostearin than to oleopalmitostearin (Lutton, 1957; Wille & Lutton, 1966). Thus by studying polymorphic forms of simple fats, glyceride configurations can be determined.

Milkfat

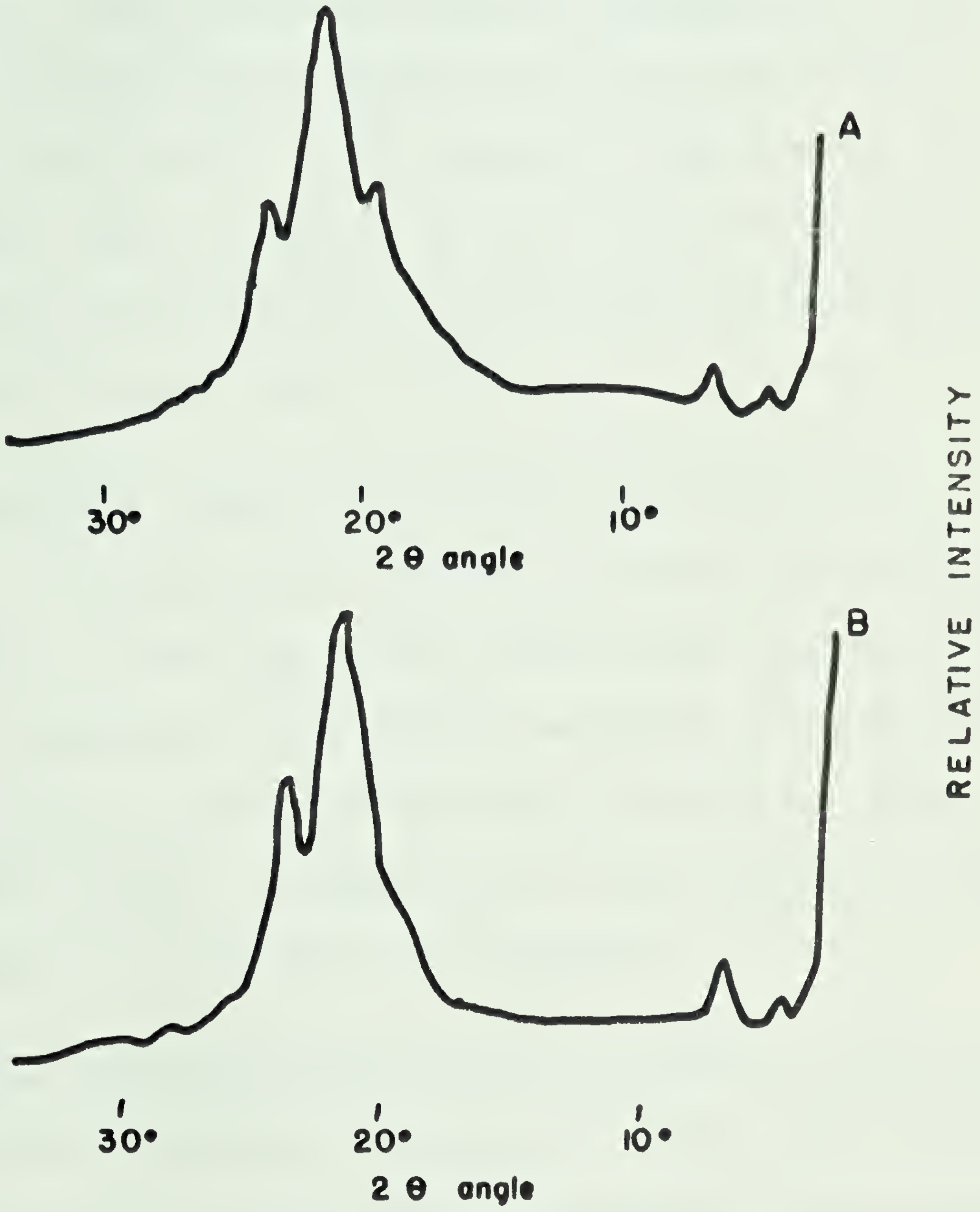
Milkfat is probably the most complicated of the natural fats. This is due to the numerous types of fatty acids that make up the glycerides. Boatman, Decoteau & Hammond (1961) reported the presence of at least 60 different fatty acids ranging from carbon number 4 to 26. The first recognition that polymorphism may occur in milkfat was obtained by Mulder (1940) who observed

two melting points of the fat, one at 19°C and the other at 30°C.

The existence of polymorphic forms of milkfat was confirmed by Greenbank (1952) and Thomas (1950). Thomas observed only two polymorphic forms of milkfat melting in the range of 18-19°C respectively, the beta-prime form had the lower melting point and the beta the higher. Tverdokhleb (1962, 1962a) obtained four polymorphic modifications of milkfat upon cooling. The gamma or glassy form was obtained from melted milkfat by rapid cooling down to below zero temperatures, melting of this form took place at 2-8°C and melting was complete at 11-14°C. By cooling the melted milkfat rapidly to 6-8°C, the alpha form was obtained. This form melts between 17.5-20°C. The beta-prime form was crystallized from melted milkfat at 15-18°C and melted at a temperature of 27-31°C. The stable beta phase was formed slowly from melted fat after a prolonged holding at 21°C and melted at 33-39°C. The evidence for the existence of these forms was based on dilatometric and heating curve studies. The gamma or glassy form has definitely been shown to be non-existent (Chapman, 1962). deMan (1961a) used X-ray diffraction to show the existence of two polymorphic forms; in milkfat the beta-prime was obtained by rapid cooling and a mixture of beta and beta-prime by slow cooling (Figure II).

The difficulty in characterizing milkfat into specific polymorphic forms arises from the diversity of glycerides present. Presence of liquid fat hinders the determination of the different polymorphic forms with X-ray diffraction. Removing the oil or simplifying milkfat by fractional crystal-

Figure II. X-ray diffraction patterns of milkfat covering the range of 2θ angles from 2 to 34°. (A) Slowly cooled; (B) rapidly cooled. From deMan (1961a).



lization into portions having various melting characteristics makes the determination possible. Conclusions based on data obtained with fractions do not necessarily apply to the whole fat.

In the high melting fraction of milkfat obtained by fractional crystallization from acetone, Thomas (1950) found three polymorphic forms, the alpha form melting at 29.3°C, the beta-prime form melting point 43.2°C, and beta form melting point 54.4°C. Four polymorphic forms were found by Beloussov & Vergelessov (1962), identical to those found by Tverdokhleb (1962), however, there is much doubt about the existence of his glassy form because of insufficient evidence (deMan, 1963).

Beloussov & Vergelessov (1966) found that the high melting point glycerides in butter continously made, transform to the beta modification. Further solidification of other high melting glycerides when butter issues from the cylinder of the butter making machine resulted in the formation of a secondary crystallization structure which forms microcleavages causing laminated and crumbling butter. To eliminate this layer formation, higher temperatures are required at the exit of the equipment.

Knoop & Samhammer (1962) maintained that the proportion of crystalline fat does not significantly influence the hardness of butter. They examined the small and very small fat crystals with X-ray diffraction and concluded that of the numerous triglycerides in milkfat only two mixed crystal fractions crystallize out, one exists of only saturated triglycerides and the other monounsaturated triglycerides. The hardness of the milkfat was

attributed to an amorphous form of solid fat. There has been no substantiated proof that an amorphous solid does exist.

It has been stated by deMan (1961) that there is no evidence linking polymorphism to physical properties of butter. This author concluded that the observed hardness changes are caused by mixed crystal formation rather than polymorphic transformations.

EXPERIMENTAL METHODS

DISTRIBUTION OF TRANS FATTY ACIDS IN MILKFAT

Source of Milkfat

The milkfat used in this work was part of a large quantity (500 lbs.) of butteroil obtained from the Agricultural Stabilization Board. It was prepared from butter made from vacreated cream by the Saskatchewan Co-op Creamery Association Limited in July, 1960. The Northern Alberta Dairy Pool prepared the milkfat in January, 1962 by passing the melted butter through a separator and a polisher, followed by treatment in a vacreator. The butter oil was then chilled, boxed and stored at -20° F.

Interesterification

The milkfat was dried at 65° C under vacuum. 0.2% (w/w) of sodium methoxide catalyst was added to the dried milkfat. The reaction was carried on in an atmosphere of inert gas with vigorous stirring for one hour at 50°C, after which the catalyst was destroyed with excess of citric acid solution. The fat was washed with warm water and dried under vacuum (deMan, 1961).

Fractional Crystallization from Acetone

The milkfat fractions were obtained by fractional crystallization from acetone (Chen, 1966). 100g of milkfat was dissolved in 1 liter

of acetone. The milkfat solution was kept at +15° overnight. The solution was filtered and the crystals washed with 200 ml acetone of the same temperature. The filtrate was made up to 1 liter volume by the addition of acetone. The filtrate was then kept at +5°C overnight. The solution was again filtered and the crystals washed with the precooled acetone. The precipitated fractions were marked +15°C and +5°C.

To collect the fractions below 0°C a special filtering apparatus was used. The device prevented temperature changes during the filtration. The apparatus consisted of an 80 mm coarse fritted glass filter with a plunger fitted with a teflon gasket. A "Minus Seventy Thermostat Bath" (Townson and Mercer, Croydon, England) kept the filtering unit at constant temperature. Crystallization was allowed to take place for 3 hours at -5°C. The filtrate was removed by applying suction to the side tube. The crystals were washed with acetone of the same temperature and then removed from the apparatus. Fractions were obtained at -15°C, -25°C, -35°C, and -45°C in a similar manner. The residue was obtained from the filtrate remaining after the -45°C fraction was collected. Interesterified milkfat was fractionated into a similar series of eight fractions using the same procedure.

Pancreatic Lipase Hydrolysis

Hydrolysis of the samples was accomplished with a procedure used by Stinson (1966). Hydrolysis was carried out in a water jacketed vessel at 37°C. The pH was maintained between 7.9 - 8.1 with a pH

controller (Fermentation Design Inc., Edison N.J.) which regulated the amount of 0.5N NaOH added (Figure III). 60 ml of water, 2 ml of 4% $CaCl_2$ solution and 15-20 mg of bile salts (Oxo Ltd., London, England) were added to the reaction vessel. Time was allowed for the solution to attain 37°C. The mixture was continuously agitated with a mechanical stirrer. The pancreatic lipase (Steapsin, Nutritional Biochemicals Corporation, Cleveland, Ohio) was added to the vessel and pH then raised to 8 by the addition of 0.5N NaOH. 2g of fat was added and the pH controller turned on. The +15°C fat fractions which are solids, were dissolved in 5 ml pentane and then added to the reaction vessel. The extent of hydrolysis was followed by the addition of the 0.5N NaOH. The volume of 0.5N NaOH required for 15% hydrolysis was calculated with the use of the saponification values. After 15% of the ester bonds of the fat samples were hydrolyzed the reaction was discontinued by the addition of 15 ml of 95% ethanol and heating to 70°C. The reaction mixture was dried in a rotary evaporator under vacuum at 70°C.

Extraction of Lipids from Hydrolyzate

Two 200 ml volumes of 50% (v/v) diethyl ether:light petroleum ether were used to extract the lipids. The ether solution was filtered over anhydrous sodium sulfate and the filtrate washed with four 50 ml portions of a 1% $NaHCO_3$ solution. The ether solution was dried by filtration over anhydrous sodium sulfate and evaporated on a steam bath.

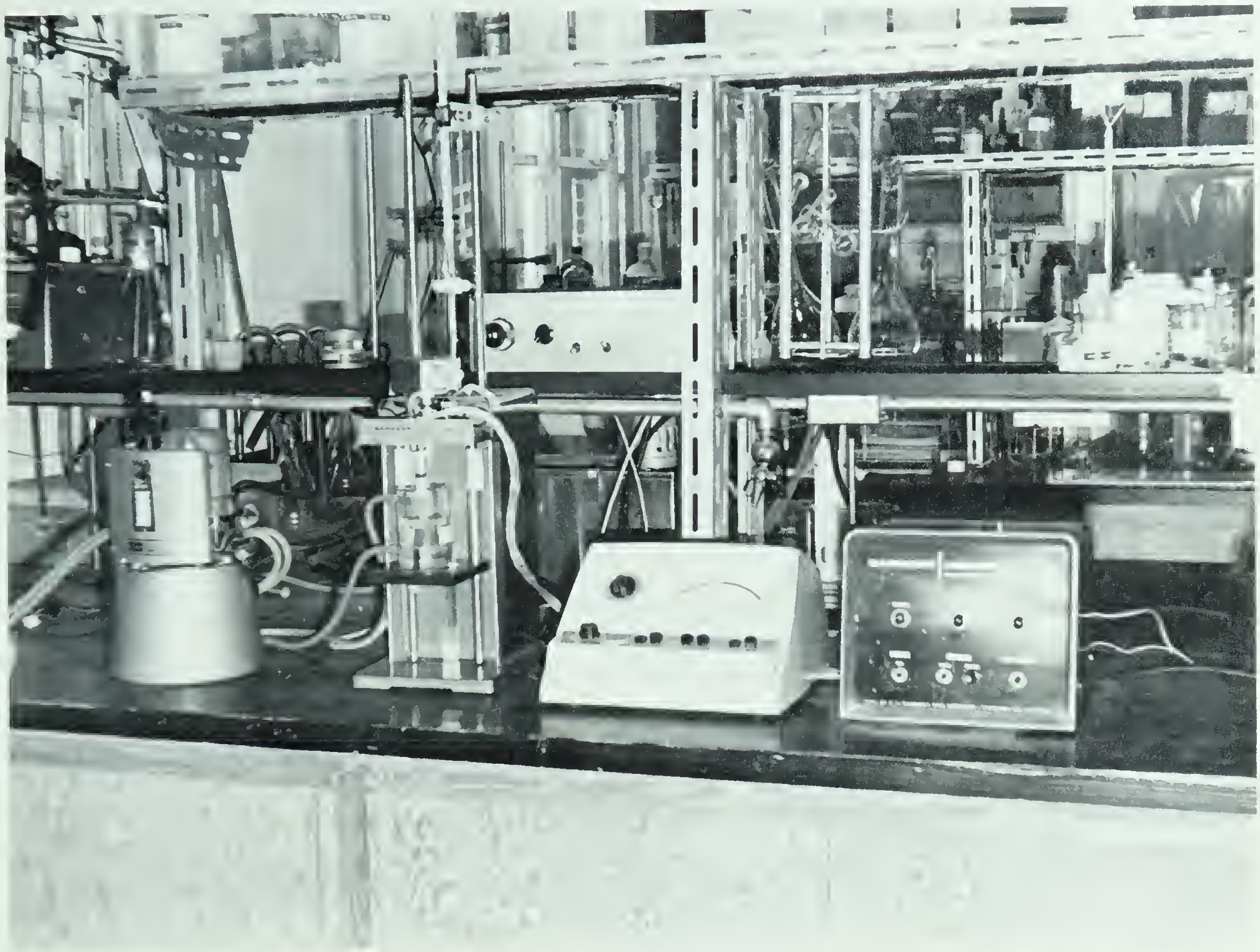


Figure III. pH controller.

Separation of Glycerides by Column Chromatography

A modified procedure similar to that of Carroll (1961) was followed for the separation of mono, di, and triglycerides. Florisil was deactivated with deionized water (7% w/v) and allowed to equilibrate for 24 hours. The columns (diameter 1.3 cm and length 22 cm) were charged with 12g of the deactivated Florisil. Florisil was packed tightly into the columns and care was exercised not to trap any air bubbles in the packing.

The hydrolyzed samples were dissolved in hexane to a concentration of 100-150 mg/ml. One ml of this solution was applied to the columns. Seven or eight columns were operated at one time (Figure IV). Two glyceride fractions were collected. The triglycerides and diglycerides were collected together in one fraction by elution with 100 ml of 50% (v/v) diethyl ether; petroleum ether solution. The monoglycerides were eluted with 100 ml of 3% (v/v) methanol in ether. The solvent was evaporated and the flask weighed.

Characterization of Material Remaining in Column

The material that remained in the column was eluted with 100 ml of methanol. The methanol was removed by evaporation on a steam bath and the residue weighed. A few mg of residue was placed between two sodium chloride discs and an infra-red spectrum between 2 μ and 15 μ was obtained.

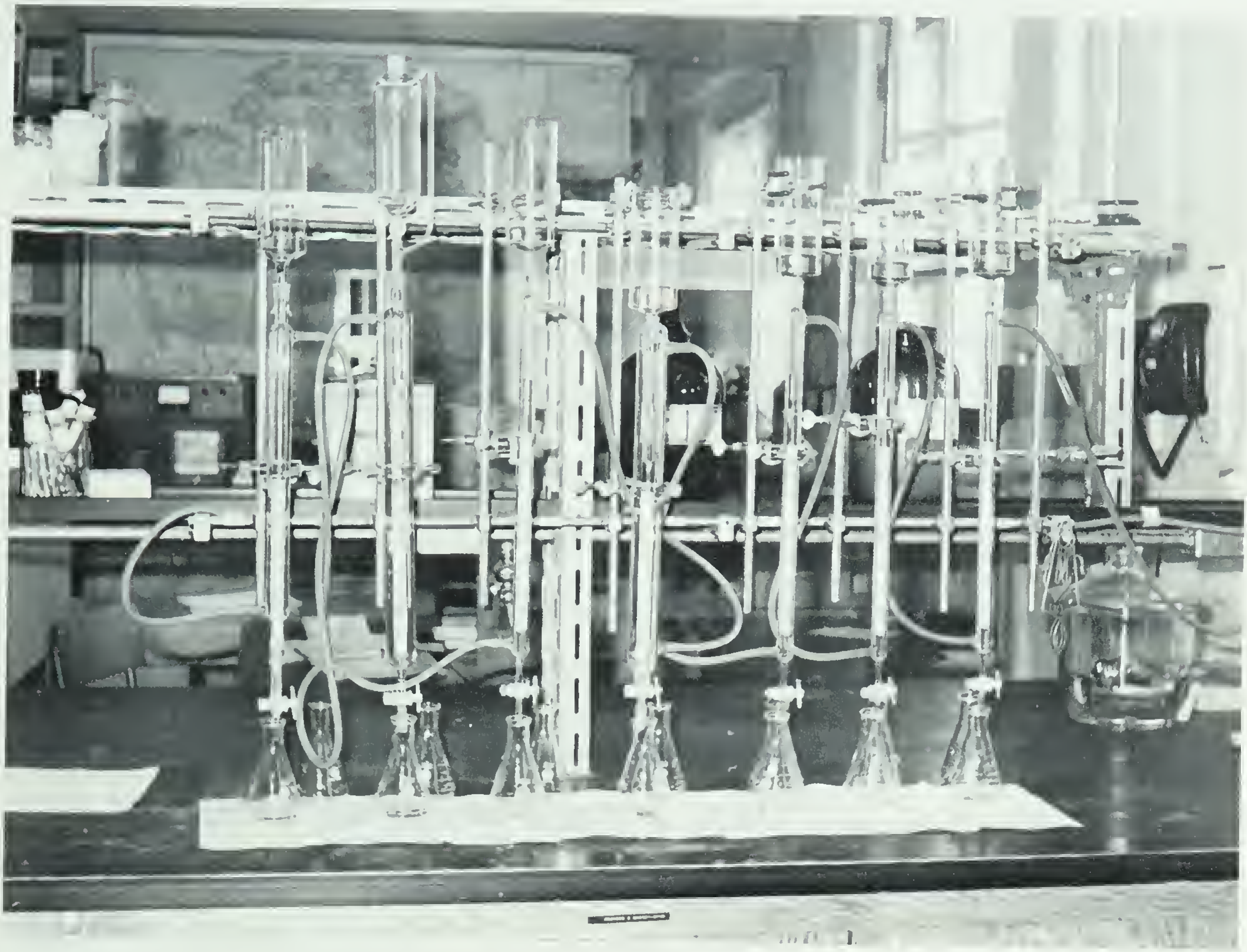


Figure IV. Column chromatography setup.

Characterization of Lipid Classes by Thin Layer Chromatography

Thin layer chromatography on Silica Gel G (Privett, Blank & Lundberg, 1961) was used to check the purity of the fractions separated by column chromatography. The thin layer plates were prepared by applying a mixture of one part silica gel G with three parts distilled water on a 20 cm x 20 cm glass plate. The thickness of the silica gel layer was 0.25 mm. The plates were dried overnight at 125°C under vacuum and stored in a cabinet containing desiccant. Before use the plates were activated by heating in an oven for at least one hour at 110°C. 10-20µl, of a 1% solution of sample was applied as a small spot before the plate was placed in the developing solvent, which consisted of light petroleum ether; diethyl ether; acetic acid (90;10:1) and was allowed to travel to within 5 cm of the top of the plate. The spots were made visible with iodine vapour (Sims & Larose, 1961).

Determination of Fatty Acid Composition

The fatty acid composition of original milkfat, interesterified milkfat, the milkfat fractions and the 2-monoglycerides obtained after hydrolysis were determined by dual column temperature programmed gas-liquid chromatography as described by deMan (1964).

Methylation was accomplished by addition of three volumes of 0.025N potassium methylate in anhydrous methanol to one part by weight of fat sample in a freeze drying bulb. The bulb was sealed and placed in an

oven at 60°C until one phase was obtained. This was a sign of complete methylation. The bulb was opened and 8-15 µl of methyl esters injected into the column of a F & M 720 dual column temperature programmed gas chromatograph. The column packing was 20% diethylene glycolsuccinate (DEGS) on a 60-80 mesh Firebrick.

Peak areas of the gas chromatograms were determined by planimetry. The correction factors used for the conversion of peak areas to weight percentage methyl esters were those determined by deMan (1964).

Determination of Trans Isomer Content

The procedure followed was that proposed in the American Oil Chemists' Society Tentative Method Cd 14-61. The % trans isomers was calculated as % trielaidin. Secondary standards were obtained from Dr. R.T. O'Connor, Chairman of the Spectroscopy Committee of the American Oil Chemists' Society.

The solid samples and standard were melted and 400-600 mg weighed accurately into glass stoppered 10 ml volumetric flasks. The volume was made up to the mark with spectrograde carbon disulfide and the solution mixed thoroughly.

A liquid cell of 0.5 mm thickness was filled with the solution and a matching cell filled with carbon disulfide. The cell containing the solvent was placed in the reference beam of a Perkin Elmer 21 double beam spectrophotometer and the cell containing sample or standard in

the sample beam. The following instrument settings were used: gain at 8, response at 1, speed at 3, suppression at 6 and rate at 2μ/min. The positions were not changed throughout the determination of trans isomers in the samples. The absorbance was measured in the region from 9 to 11μ.

For determination of the absorbance at 10.36μ a base line was drawn from 10.05μ to 10.67μ (a to b in Figure V) on the absorption curves. The absorbance of the sample was read from the maximum absorbance at 10.36μ to the base line (d minus c in Figure V). From Beer's and Lambert's Law, the determination of % trans as trielaidin was calculated from the following formula:

$$\%\ \underline{\text{trans}}\ \text{as trielaidin} = \frac{a \quad \text{sample}}{a \quad \text{trielaidin standard}} \times 100$$

where: a = absorptivity = A/bc

and: A = absorbance

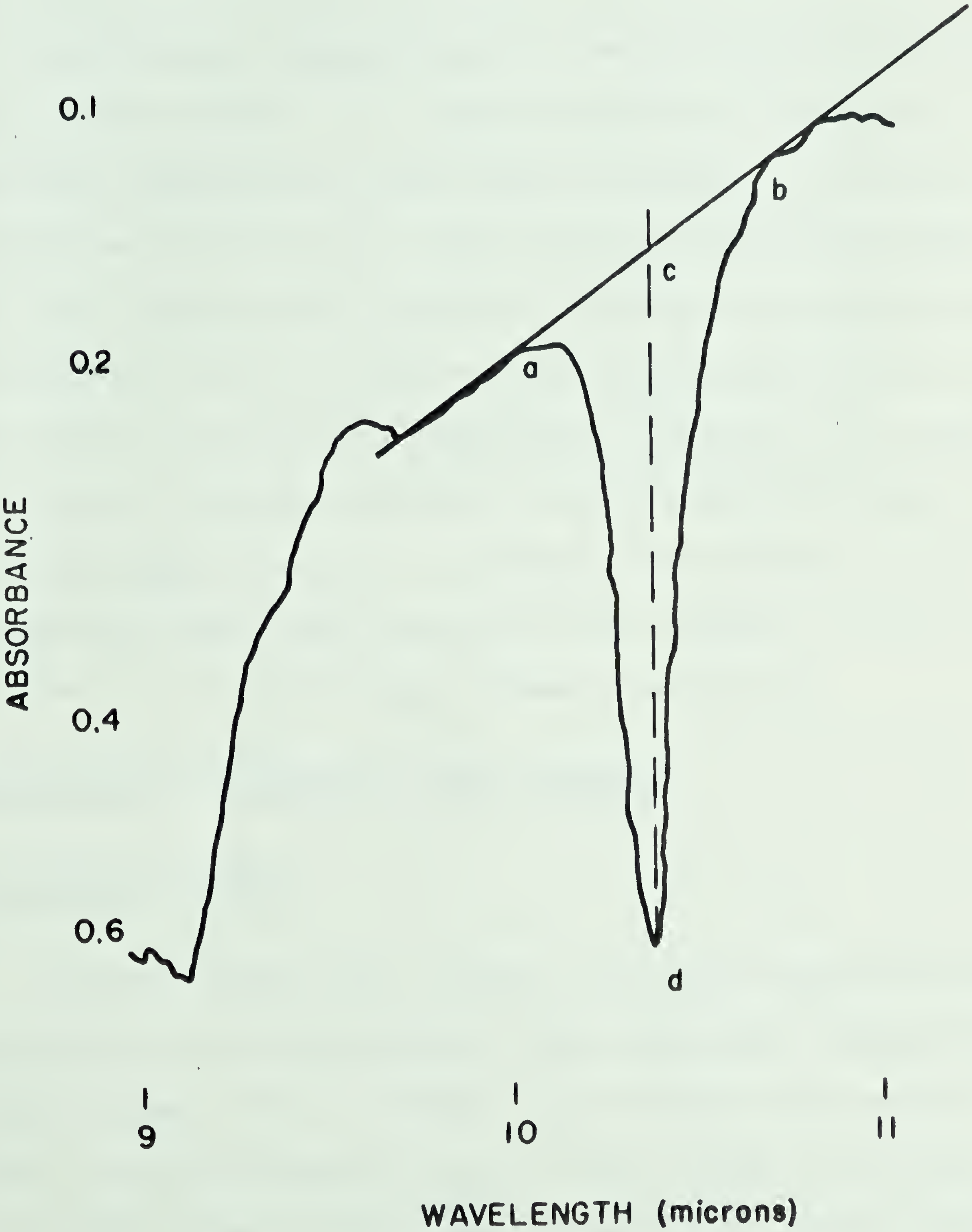
b = liquid cell thickness (cm)

c = concentration of solution (g/l)

Determination of Percent Trans as Trielaidin in 2-Monoglycerides from Hydrolyzate

A modification of the American Oil Chemists' Society procedure Cd 14-61 for the determination of percent trans as trielaidin was required for the 2-monoglycerides. The reason was the insolubility of the monoglycerides in carbon disulfide.

Figure V. Determination of percentage trans unsaturation from the height of the peak at 10.36 μ.



Trielaidin (Nutritional Biochemical Corporation, Cleveland, Ohio) was used in a control experiment. The percentage trans in the 2-monoglycerides was determined first by the method previously described. Since only approximately 0.2 g of 2-monoglycerides were collected the sample was diluted to only 5 ml with carbon disulfide.

The detection of trans isomers in 2-monoglycerides involved the sample without dilution. The sample was melted and a few drops of the liquid 2-monoglycerides were put on a NaCl disc. A tin spacer (0.012mm) was placed over the sample and then covered with another NaCl disc. The discs were placed in a cell mount where slight pressure was applied so that the thickness of the sample between the two plates was uniform. The spectrum between 9μ and 11μ was run. If an absorption peak at 10.36μ was prominent, it was concluded that there was sufficient trans isomers for a quantitative determination. All 2-monoglyceride samples were subjected to this procedure to see if it was necessary to determine trans content quantitatively.

DETERMINATION OF POLYMORPHIC FORMS OF MILKFAT

Infra-red Spectroscopy

The region between 770 - 690 cm^{-1} of the infra-red spectrum has been used for the characterization of the polymorphic configuration of fats (Chapman, 1965). Different heat treatments were applied to milkfat and the high melting fraction (HMF) obtained by crystallization at 15°C. The treatments were carried out in a Variable Temp-

erature Chamber Model 104 (Barnes Engineering Company). The spectra were obtained with a Perkin Elmer 21 infra-red spectrophotometer. The experimental set-up is shown in Figure VI.

Heat treatments applied to the samples involved slow cooling or fast cooling. The milkfat was warmed to 40°C and a few drops of the liquid fat transferred to a NaCl disc, covered by a second disc and placed in a cell holder. The cell holder was put in the Variable Temperature Chamber. The temperature in the chamber was raised to 40°C and a spectrum obtained. The temperature was then lowered by 4°C per hour and a spectrum run every hour until a temperature of 0°C was reached. Lower temperatures caused fogging of the NaCl windows of the Variable Temperature Chamber. The wavenumbers of the maximum absorption peaks were recorded. For the slow cooling treatment of the HMF, the temperature was raised to 60°C, and cooled at 4°C per hour until a temperature of 10°C was reached.

For the fast cooling treatment of milkfat the temperature was dropped rapidly from 40°C to 0°C in the chamber and held at 0-3°C for 23 hours. A spectrum was run after the temperature reached 0°C and after the 23 hour period. The HMF was cooled rapidly from 60 to 7°C. Spectra were run continuously for the first 15 minutes and after 20 hours. The wave numbers of the maximum absorption peaks were recorded.

The HMF obtained by fractional crystallization from acetone was

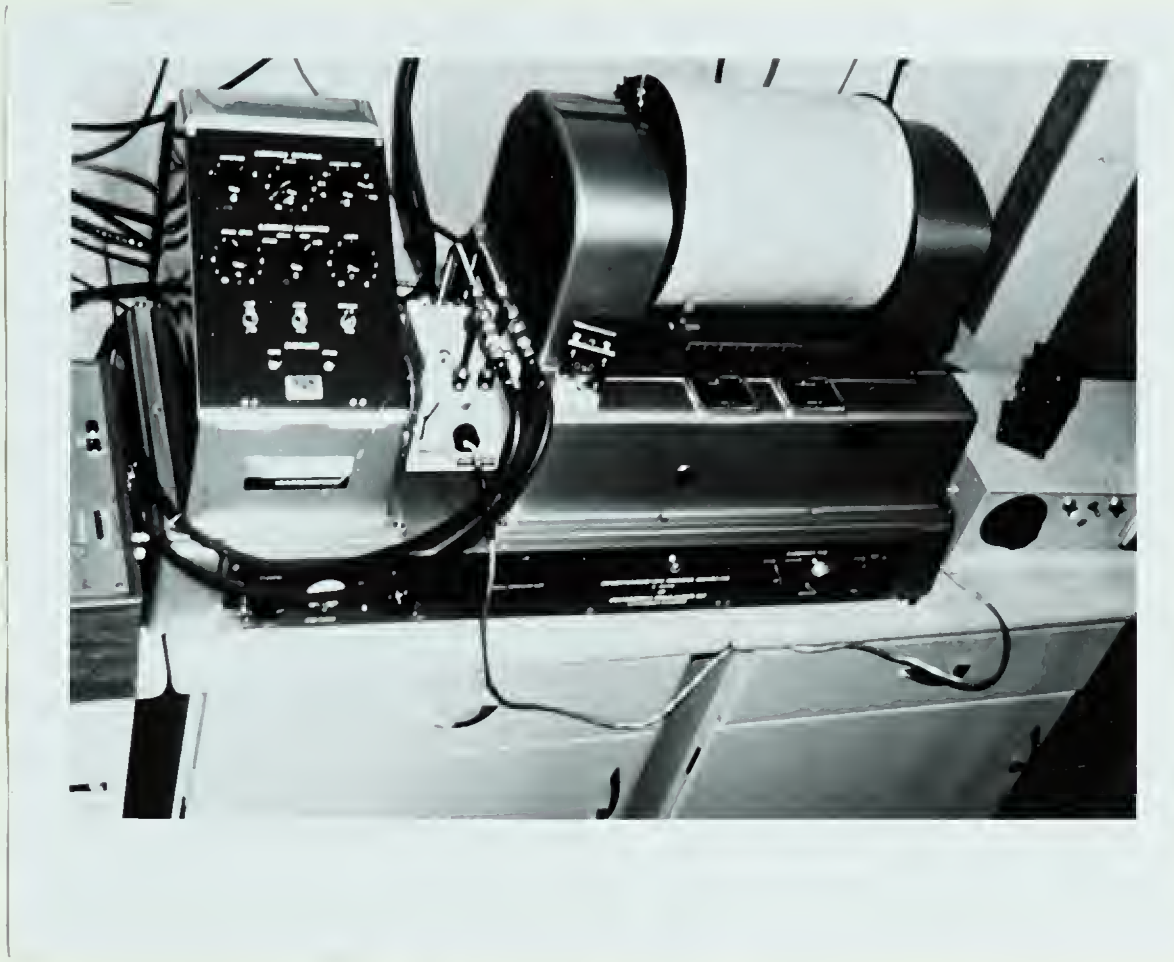


Figure VI. Perkin Elmer 21 infra-red spectrophotometer.

placed between two NaCl discs and the infra-red spectrum between 770 and 690 cm^{-1} was determined.

X-ray Diffraction

A Philips Geiger counter diffractometer was the instrument used to obtain the X-ray diffraction patterns between 2θ angles of 15° and 30°. The samples were melted and filled into aluminum cell mounts. The treatments included fast cooling and slow cooling. Whole milkfat was rapidly cooled by heating the cell mount to 40°C and then placing the mount in a Petri dish surrounded by dry ice. The X-ray shield was packed with dry ice to maintain the temperature at about 0°C while the pattern was obtained. Before placing the sample in the X-ray beam the surface of the sample was smoothed with a spatula. The radiation used was Cu, $K\alpha$ ($\lambda = 1.5405$ Å). The slowly cooled sample was prepared by placing the cell mount in the Variable Temperature Chamber and cooling from 40°C to 0°C in 24 hours.

The high melting fraction (HMF) was obtained by fractional crystallization of milkfat from acetone at +15°C. The HMF fraction that was obtained was filled in a cell mount and the X-ray diffraction pattern ran. The HMF in the cell mount was heated to 65°C in the Variable Temperature Chamber and cooled rapidly to 10°C. The X-ray diffraction pattern was obtained at room temperature. A slowly cooled treatment of the HMF was accomplished by placing the cell mount in the Variable Temperature Chamber and slowly cooling at 4°C per hour

from 65°C to 10°C.

To illustrate the effect of the presence of oil on the quality of X-ray diffraction pattern, the following procedure was followed. 100 g of milkfat was heated to 40°C until completely melted. A portion of the melted milkfat was poured into a cell mount. The cell mount and the beaker of melted fat was allowed to remain at room temperature for 24 hours. The solid high melting fraction from the beaker was removed by pressure filtration (Vasic & deMan, 1966). A cell mount was packed with this high melting fraction and the diffraction pattern obtained at room temperature. A diffraction pattern of milkfat tempered at room temperature was also determined. The X-ray diffraction angles were converted to interplanar spacings of all the diffraction patterns obtained (National Bureau of Standards, 1950).

RESULTS

TRANS FATTY ACID DISTRIBUTION IN MILKFAT, INTERESTERIFIED MILKFAT AND THEIR FRACTIONS

Fractional crystallization

The milkfat and interesterified milkfat were fractionated by solvent crystallization from acetone and eight fractions were obtained from each product. The fatty acid composition of the milkfat and its fractions are listed in Table VI. The fatty acid composition of interesterified milkfat and its fractions are given in Table VII. The concentration of palmitic acid in the original milkfat fraction decreased from 41.9% in the +15°C fraction to 11.2% in the residue. Stearic acid decreased as the temperature at which the fractions were obtained decreased, from 30.2% in the +15°C fraction to 1.6% in the residue. The concentration of oleic acid increased from 11.6% in the +15°C fraction to 47.8% in the residue. The concentration of short chain fatty acids, butyric and caproic increased by 10% while the other fatty acids did not vary in concentrations in the milkfat fractions. The interesterified fractions also showed similar trends; palmitic acid decreased from 46.9% in the 15°C fraction to 10.6% in the residue, stearic acid similarily decreased from 26.5% to 1.7%, oleic acid increased from 8.9% to 43.7% and butyric acid increased from a trace to 11.0%

Saponification Values of Milkfat, Interesterified Milkfat and their Fractions

The average molecular weight of the fatty acids in the milkfat,

interesterified milkfat and their fractions were calculated from the concentrations of the specific methyl esters in the sample (Table VI and Table VII). The formula

Saponification Value = 56,100/Average Molecular Weight

was used for the calculations. The saponification values and the average molecular weights of the samples are listed in Table VIII. The saponification values of the milkfat fraction increased from 202.4 for the +15°C fraction to 218.9 for the residue similarly the corresponding values for the interesterified milkfat were 203.6 and 227.3. The presence of increasing amounts of short chain fatty acids results in an increase in saponification values.

Chromatographic Separation of Partial Glycerides in Hydrolyzate

The completion of 15% hydrolysis of the samples was determined by the amount of 0.5N NaOH added to the reaction vessel by the pH controller. The amount of NaOH required was previously calculated with the use of the saponification values of Table VIII. As an example of the calculation, first, the number of milliequivalents of KOH needed for complete saponification is determined from saponification value/ 56.1. The number of milliequivalents for 15% hydrolysis is then 15% of the total milliequivalents. This value is then multiplied by 2 to give the number of ml of 0.5N NaOH needed for 15% hydrolysis of 1g of fat.

After the hydrolyzate was purified, a known amount was placed on

a Florisil column. The glycerides recovery was determined by the weight of the fractions collected. The percentage of monoglycerides recovered are listed in Table IX. It was found that after 15% hydrolysis, approximately 30% monoglycerides were recovered from the total glycerides eluted from the column, however, the total recovery of the lipid material applied to the columns was 65%. In a control experiment, where trielaidin was separated into mono, di, and triglycerides, the yield was 100%. The trielaidin used consisted of 92.7% triglycerides, 5.2% diglycerides and 2.1% monoglycerides. The 100% recovery indicated that no loss of the mono, di, and triglycerides occurred during the separation by column chromatography.

Characterization of Material Remaining in Column

Since the yield of the lipid material eluted from the column was only 65%, 35% of material must have remained in the column. When the column was eluted with 100 ml of methanol, a fraction was obtained that brought the yield to 100%. A few mg of the residue were placed between two NaCl discs and an infra-red spectrum obtained (Figure VII). From the spectrum it was concluded that the residue was the sodium salt of long chain carboxylic acids.

Purity Check of Partial Glycerides by Thin Layer Chromatography

To check the purity of the partial glycerides recovered from Florisil, thin layer chromatography on Silica gel G was used. This procedure was done only to insure that the monoglycerides recovered from the column

were pure. An illustration of a thin layer plate appears in Figure VIII. This chromatogram shows that the partial glycerides were separated into two fractions, one fraction containing di- and triglycerides and few impurities and the other fraction containing pure monoglycerides.

Percentage Trans Fatty Acids (as Trielaidin) in Milkfat, Interesterified Milkfat and their Fractions

The percentage of trans fatty acids, unsaturation and the ratio of trans fatty acids to total unsaturated fatty acids in the milkfat, interesterified milkfat and their fractions are listed in Table X. The standard used contained 24.1 ±1.0% trans as trielaidn and was obtained from Dr. R.T. O'Connor of the Spectroscopy Committee of the American Oil Chemists' Society. It was found that in the +15°C fraction of the original fat, 81.6% of the unsaturated fatty acids were trans while 69.0% of the unsaturated fatty acids in the interesterified +15°C fraction were trans. The ratio of trans to unsaturation decreased as the temperature of crystallization decreased. The ratio of trans in fractions of original milkfat decreased from 35.3% in the +5°C fraction to 15.8% in the residue. The ratio of trans in the interesterified fractions decreased from 34.7% in the +5°C fraction to 19.0% in the residue.

Trans Determination in 2-Monoglycerides

Approximately 20 mg of the 2-monoglycerides collected were placed between two NaCl discs containing a spacer of 0.012 mm thickness and a

spectrum obtained between 9μ and 11μ. The spectrum was that of solid or crystalline monoglycerides, and was different from that obtained when the sample was in solution (Figure IX). The spectrum of the crystalline 2-monoglycerides showed a small shoulder in the region of 10.36μ. The -15°C 2-monoglyceride fraction of milkfat exhibited the largest absorption of all the fractions in the region of 10.36μ. This fraction was made into a 4% solution in CS_2 and a spectrum run between 9μ and 11μ. The solution spectrum had an indication of a shoulder present at 10.36μ but too small to determine the trans content quantitatively. The spectra of all 2-monoglycerides collected after hydrolysis of the milkfat fractions did not indicate the presence of measurable amounts of trans fatty acids.

In the control experiment, a spectrum (Figure X) of the 2-monoglycerides neat obtained from the hydrolyzate after pancreatic hydrolysis of trielaidin was run. A prominent absorption band at 10.36μ appeared. A solution of 2-monoglycerides in CS_2 was made and a spectrum obtained and the % trans (as trielaidin) calculated. It was found that 68.0% trans as trielaidin in the monoglycerides.

Fatty Acid Composition of 2-Monoglycerides

The 2-monoglycerides were converted to their methyl esters by the method of deMan (1964). A gas-liquid chromatogram was obtained of the methyl esters. Figure XIA represents the gas chromatogram of the +15°C fraction of milkfat. The gas chromatograph was programmed to operate from 70°C to 220°C at 7.5°/ min. The chromatogram of Figure XIB represents

the methyl esters of the 2-monoglycerides of the +15°C original fraction after hydrolysis. In this case the gas chromatograph was run isothermally at 220°C. The percentages of the fatty acids found in the 2-monoglycerides of milkfat, interesterified milkfat and their fractions are listed in Tables VI and VII. The percentage of fatty acids esterified in the 2-position are given in Tables XI and XII. The calculations were made with the following formula (McCarthy, Patton & Evans, 1960).

$$\%\ \text{fatty acid in 2-position} = \frac{\%\ \text{of a fatty acid in the monoglycerides formed}}{\%\ \text{of the same fatty acid in the original triglyceride} \times 3} \times 100$$

The interesterified milkfat had a more random distribution of fatty acids than the original but the randomization appeared not to be complete. This can be seen by the fact the fatty acids are not equally distributed among the glycerides i.e. 33.3% fatty acid in the 2-position. Table XII shows that in the original milkfat the fatty acids that occur in random distribution are capric and the fatty acids that are classed in group B. The fatty acids lauric, myristic, group A, and palmitic are mainly in the 2-position while the rest of the fatty acids are predominantly in the 1 and 3 positions of the glyceride molecule.

The fatty acid composition of milkfat, interesterified milkfat and their fractions are compared in Table XIII. It appears that the interesterified milkfat and its fractions have a greater proportion of stearic acid in the 2-position that the original fat while the original fat and its fractions have a greater proportion of lauric and myristic acids in

the 2-position.

POLYMORPHISM

Milkfat

Figure XII represents the infra-red spectra between 770 - 690 cm^{-1} of milkfat before and after rapid cooling. Spectrum XIIA is of liquid milkfat at 40°C, and has a peak maximum at 720 cm^{-1}. As the milkfat was cooled rapidly from 40 to 0°C in the Variable Temperature Chamber at 1°C/sec the absorbance of 720 cm^{-1} increased (Figure XIIB). After the sample had reached 0°C, the temperature was held at 5°C for 10 hours and at that time another spectrum was obtained (Figure XIIC). Maximum absorption peaks occurred at 718 cm^{-1} and a strong shoulder at 727 cm^{-1}.

Milkfat was cooled slowly by decreasing the temperature in the Variable Temperature Chamber at 4°C per hour. Figure XIII shows the spectra at 4°C intervals until a low of 0°C was reached. The peak maximum at 720 cm^{-1} of the melt gradually shifted and formed a strong peak at 718 cm^{-1} and shoulder at 727 cm^{-1} at 0°C.

The X-ray diffraction patterns of both slowly and rapidly cooled milkfat were similar (Figures XIV and XV). The short spacings of the slowly cooled fat were found to be 4.64, 4.17 and 3.78 Å and the

rapidly cooled fat 4.57Å, 4.11Å, 3.83 Å. The infra-red spectra and X-ray diffraction patterns indicate that the beta, beta-prime and alpha forms were present.

High Melting Fraction of Milkfat

The high melting fraction (HMF) obtained by fractional crystallization from acetone was cooled rapidly from the melt at 65°C to 10°C. The infra-red spectrum obtained with the melt (Figure XVIA) showed a broad peak maximum at 720 cm^{-1}. The spectrum of the solid, at 10°C had an absorption maximum at 720 cm^{-1} (Figure B). The short spacings calculated from the X-ray diffraction pattern of the rapidly cooled HMF were 4.11 Å and a weak spacing at 3.85 Å (Figure XVII), this indicates the presence of the alpha polymorph.

Slow cooling of the HMF from 60°C to 10°C resulted in gradual conversion of the broad absorption peak at 720 cm^{-1} to a prominent doublet at 727 cm^{-1} and 718 cm^{-1} (Figure XVIII), this is an indication of the formation of the beta-prime polymorphic form. This was confirmed by the X-ray diffraction pattern (Figure XIX) from which short spacings of 4.23Å, 4.13Å (w) and 3.84Å were calculated.

The HMF obtained after crystallization from acetone was used to determine the polymorphic form without previous heat treatments. The infra-red spectrum between 770 cm^{-1} and 690 cm^{-1} showed an absorption band at 717 cm^{-1} (Figure XX). The presence of the singlet at 717 cm^{-1} and the X-ray diffraction pattern with short spacings at 4.61Å, 3.88Å,

3.75Å and 3.60Å (Figure XXI) indicated the presence of the beta modification. A summary of the different sample treatments and their effect on the form of infra-red spectra and X-ray diffraction patterns is given in Table XIV.

To illustrate the effect of the presence of relatively large amounts of liquid oil on the resolution of the X-ray diffraction patterns, a pattern of milkfat was obtained at room temperature (Figure XXII. The solid portion of this fat at the same temperature was obtained by pressure filtration and the crystals packed in the cell mount. The pattern was run between 2 θ angles of 15° and 30° (Figure XXIII). The diffraction pattern of the whole milkfat is broad and diffuse, whereas the pattern obtained with the crystals is much better defined.

Table VI. Fatty acid composition of milkfat, its fractions and the 2-monoglycerides obtained by pancreatic lipase hydrolysis.

Fraction	Fatty acids													
	4:0	6:0	8:0	10:0	10:1	12:0	14:0	A[a]	16:0	B[b]	18:0	18:1	18:2	18:3
Whole	3.8	1.8	1.0	2.1	0.2	2.3	9.1	2.8	27.1	3.8	12.8	30.6	1.6	1.1
2-Mono	-	-	0.3	2.2	-	3.6	17.3	3.2	37.9	3.2	7.1	24.8	0.4	-
+ 15	0.2	tr.	tr.	0.6	-	1.6	10.1	1.8	41.9	2.0	30.2	11.6	tr.	tr.
2-Mono	-	-	-	0.3	-	1.2	15.9	1.8	61.2	0.5	15.9	3.1	-	-
+ 5	2.8	1.4	0.6	1.6	tr.	2.1	8.8	2.3	35.4	2.2	20.9	21.7	0.2	tr.
2-Mono	-	tr.	0.2	1.3	-	2.0	15.7	1.7	51.1	1.2	13.8	13.0	-	-
- 5	4.0	1.9	1.0	1.8	0.3	1.8	9.9	2.3	36.0	2.1	15.3	22.8	0.5	0.1
2-Mono	-	-	-	0.9	-	1.9	21.2	1.9	51.3	0.6	9.4	12.8	-	-
- 15	3.6	1.8	0.9	1.9	0.2	2.6	11.2	3.1	28.9	3.7	11.3	29.4	0.8	0.4
2-Mono	-	-	0.2	2.0	-	4.0	22.2	2.4	38.8	2.1	5.8	22.6	-	-
- 25	3.8	2.0	1.1	2.5	0.2	3.1	9.1	3.4	20.6	3.5	7.3	39.0	2.1	1.0
2-Mono	-	-	1.0	3.4	0.3	5.6	16.1	3.9	25.7	5.0	2.8	33.9	1.4	-
- 35	3.9	2.3	1.2	2.8	0.2	2.9	7.5	2.9	19.5	4.1	7.3	42.0	1.6	1.3
2-Mono	-	-	1.1	3.2	0.3	4.9	12.1	4.4	20.1	5.5	1.9	42.0	3.2	0.5
- 45	5.7	3.2	1.6	2.5	0.3	3.1	7.5	2.8	18.0	3.8	5.4	42.0	1.9	1.7
2-Mono	-	-	1.1	3.8	0.3	4.8	12.0	3.6	22.4	5.6	3.3	39.9	2.5	-
Residue	8.2	3.4	1.7	2.8	0.4	2.6	7.2	2.5	11.2	4.5	1.6	47.8	2.4	2.4
2-Mono	tr.	0.2	1.8	5.0	0.2	3.6	12.1	4.4	20.4	5.5	1.7	41.0	3.2	0.2

a. 14:1, 15:0, $15{:}0^{iso}$
b. 16:1, 17:0, $17{:}0^{iso}$

Table VII. Fatty acid composition of interesterified milkfat, its fractions and the 2-monoglycerides obtained by pancreatic lipase hydrolysis.

Fraction	Fatty acids													
	4:0	6:0	8:0	10:0	10:1	12:0	14:0	A[a]	16:0	B[b]	18:0	18:1	18:2	18:3
Whole	3.8	1.7	0.9	1.9	0.2	2.1	9.0	3.4	27.0	4.5	12.6	30.4	1.4	0.9
2-Mono	-	-	1.0	2.7	-	2.8	11.2	1.3	34.5	1.6	15.4	29.6	-	-
+ 15	0.1	tr.	tr.	0.4	-	1.5	11.1	2.1	46.9	2.2	26.5	8.9	0.2	tr.
2-Mono	-	-	tr.	tr.	-	0.5	11.0	0.9	53.0	0.2	30.5	4.0	-	-
+ 5	1.2	0.8	0.6	1.5	0.1	2.8	11.9	2.7	35.2	2.3	17.1	23.0	0.6	tr.
2-Mono	-	-	-	1.2	-	2.2	13.8	1.2	44.1	0.3	22.1	15.2	-	-
- 5	1.6	1.1	0.6	1.8	0.2	2.2	9.1	2.4	33.1	2.4	17.8	26.7	0.6	0.2
2-Mono	-	-	-	2.1	-	3.2	10.9	0.6	40.7	tr.	18.2	24.2	-	-
- 15	2.8	1.5	0.9	1.9	0.1	2.4	9.5	2.7	29.3	2.9	13.8	30.7	0.8	0.6
2-Mono	0.1	0.2	0.7	2.2	0.2	2.6	11.2	2.6	32.1	1.3	13.4	33.4	-	-
- 25	3.5	1.8	1.0	2.3	0.1	2.6	9.4	3.0	23.6	4.1	9.4	36.1	1.9	0.9
2-Mono	tr.	0.1	0.7	2.6	0.2	2.7	11.3	2.9	28.8	3.6	11.3	34.3	1.0	-
- 35	4.4	2.3	1.2	2.5	0.1	2.7	8.8	3.4	20.4	4.6	8.0	38.0	2.2	1.2
2-Mono	-	-	1.1	3.3	-	3.2	11.5	2.5	23.9	3.0	7.6	43.7	0.2	-
- 45	5.8	3.2	1.6	2.8	0.4	3.4	8.5	2.7	18.3	4.8	6.7	38.0	1.7	1.6
2-Mono	tr.	0.1	1.5	3.0	0.3	3.6	9.6	5.6	20.2	4.5	7.3	41.4	2.1	0.4
Residue	11.0	5.4	2.1	3.5	0.4	3.0	7.0	2.7	10.6	4.0	1.7	43.7	2.4	1.6
2-Mono	0.6	2.2	3.0	4.7	0.3	3.9	8.4	3.8	14.3	4.3	2.5	50.5	1.5	-

a. 14:1; 15:0; $15{:}0^{\mathrm{iso}}$
b. 16:1; 17:0; $17{:}0^{\mathrm{iso}}$

Table VIII. Saponification values of milkfat, interesterified milkfat and their fractions calculated from the average molecular weight of the fatty acids.

Fraction	Average Molecular Weight		Saponification Value[a]	
	Original	Interesterified	Original	Interesterified
Whole	267	267	210	210
+15	277.2	275.6	202.4	203.6
+ 5	269.5	270.8	208.2	207.2
- 5	264.4	271.9	212.2	206.3
-15	265.0	268.8	211.7	208.7
-25	265.8	267.0	211.1	210.1
-35	265.9	264.6	211.0	212.0
-45	260.9	259.4	215.0	216.3
Residue	256.3	246.8	218.9	227.3

a. Saponification Value = 56,100 / Average Molecular Weight

Table IX. Percentage monoglycerides recovered from the hydrolysis of milkfat, interesterified milkfat and their fractions.

Fraction	Percentage Monoglycerides in Hydrolyzate		Percentage Monoglycerides in Glyceride Recovery	
	Original	Interesterified	Original	Interesterified
Whole	27.8	22.2	39.6	32.7
+15	12.0	17.8	18.6	29.0
+ 5	24.6	22.5	38.0	43.1
- 5	20.9	18.0	31.5	32.2
-15	21.1	18.0	29.8	26.3
-25	21.8	16.3	30.9	23.6
-35	30.1	17.4	53.8	24.3
-45	22.6	20.1	34.3	30.1
Residue	18.9	12.6	29.6	20.1

Table X. Percentage *trans* and unsaturated fatty acids and their ratio in milkfat, interesterified milkfat and their fractions.

Fraction	Trans		Unsaturation[a]		Trans/Unsaturation[b]	
	Orig	Inter	Orig	Inter	Orig	Inter
Whole	8.6	8.8	37.2	37.4	23.1	23.5
+15	11.1	7.8	13.6	11.3	81.6	69.0
+ 5	8.5	9.0	24.1	25.9	35.3	34.7
- 5	8.1	7.5	25.8	30.1	31.4	24.9
-15	9.9	9.0	34.5	35.1	28.7	25.6
-25	9.8	9.8	45.6	43.1	21.5	22.7
-35	9.2	9.7	49.2	46.1	18.7	21.0
-45	9.0	9.4	49.7	46.5	18.1	20.2
Residue	9.1	9.9	57.5	52.1	15.8	19.0

a. Unsaturation includes fatty acids 10:1, B, 18:1, 18:2, 18:3.

b. Percentage ratio of *trans* content over total unsaturation.

Table XI. Percentage fatty acid esterified in 2-position of milkfat and its fractions.[a]

Fraction	Fatty Acid										
	8:0	10:0	10:1	12:0	14:0	A	16:0	B	18:0	18:1	18:2
Whole	11.3	34.9	-[b]	52.2	63.4	50.0	46.6	28.1	18.5	27.0	8.3
+15	-	16.7	-	25.0	52.5	33.3	48.7	9.5	17.5	8.9	-
+ 5	11.1	27.1	-	31.7	59.5	24.6	48.1	18.2	22.0	20.0	-
- 5	-	16.7	-	35.2	71.4	27.4	27.5	47.5	9.5	20.5	-
-15	7.4	35.1	-	51.3	66.1	25.8	44.8	18.9	17.1	25.6	-
-25	30.3	45.3	50.0	60.2	59.0	38.2	41.6	47.6	12.8	29.0	22.2
-35	30.6	38.1	50.0	56.3	53.8	50.6	34.4	44.7	8.7	33.3	66.7
-45	22.9	50.7	33.3	51.6	53.3	42.8	41.5	49.1	20.4	31.7	43.8
Residue	35.3	59.5	16.7	46.2	56.0	58.7	60.7	40.7	35.4	28.6	44.4

a. Calculated from $\frac{M}{3T} \times 100$ where M = mole percent of the acid in the monoglycerides
T = mole percent of the same acid in the triglycerides before hydrolysis.

b. Amounts of fatty acids in triglycerides and monoglycerides too low for reliable calculation of proportion present at 2-position.

Table XII. Percentage fatty acid esterified in 2-position of interesterified milkfat and its fractions.[a]

Fraction	8:0	10:0	10:1	12:0	14:0	A	16:0	B	18:0	18:1	18:2
Whole	37.0	53.7	-[b]	44.4	41.5	12.7	42.6	11.8	40.7	40.7	-
+15	-	-	-	11.1	33.0	14.3	37.7	3.0	38.4	15.0	-
+ 5	-	26.7	-	26.2	38.6	14.8	41.8	4.3	43.1	22.0	-
- 5	25.9	38.9	-	48.5	39.9	8.3	41.0	-	34.1	30.2	-
-15	25.9	38.6	66.7	36.1	39.3	32.1	36.5	14.9	32.4	36.3	-
-25	23.3	37.7	66.7	34.6	40.1	32.2	40.7	29.3	40.1	31.7	17.5
-35	30.6	44.0	-	39.5	43.6	24.5	39.0	21.7	31.7	38.3	30.3
-45	31.2	35.7	25.0	35.3	37.6	69.1	36.8	31.2	36.3	36.3	41.2
Residue	47.6	44.8	25.0	43.3	40.0	46.9	46.9	35.8	49.0	38.5	20.8

a. Calculated from $\frac{M}{3T}$ x 100 where M = mole percent of the acid in the monoglycerides
T = mole percent of the same acid in the triglycerides before hydrolysis.

b. Amounts to fatty acids in triglycerides and monoglycerides too low for reliable calculation of proportion present at 2-position.

Table XIII. Fatty acids that exceed 10% difference in the percentage fatty acids in the 2-position of milkfat, interesterified milkfat and their fractions.

Fraction	Fatty Acids										
Whole	$\underline{8:0}$[a]	$\underline{10:0}$			14:0	A		B	$\underline{18:0}$		
+15		10:0		12:0	14:0	A	16:0		$\underline{18:0}$		
+ 5	8:0				14:0			B	$\underline{18:0}$		
- 5		$\underline{10:0}$		12:0	14:0	A		B	$\underline{18:0}$	$\underline{18:1}$	
-15	$\underline{8:0}$		$\underline{10:1}$	12:0	14:0				$\underline{18:0}$	$\underline{18:1}$	
-25			$\underline{10:1}$	12:0	14:0			B	$\underline{18.0}$		
-35			10:1	12:0	14:0	A		B	$\underline{18:0}$		18:2
-45		10:0		12:0	14:0	$\underline{A}$		B	$\underline{18:0}$		
Residue	$\underline{8:0}$	10:0			14:0	A	16:0		18:0		18:2

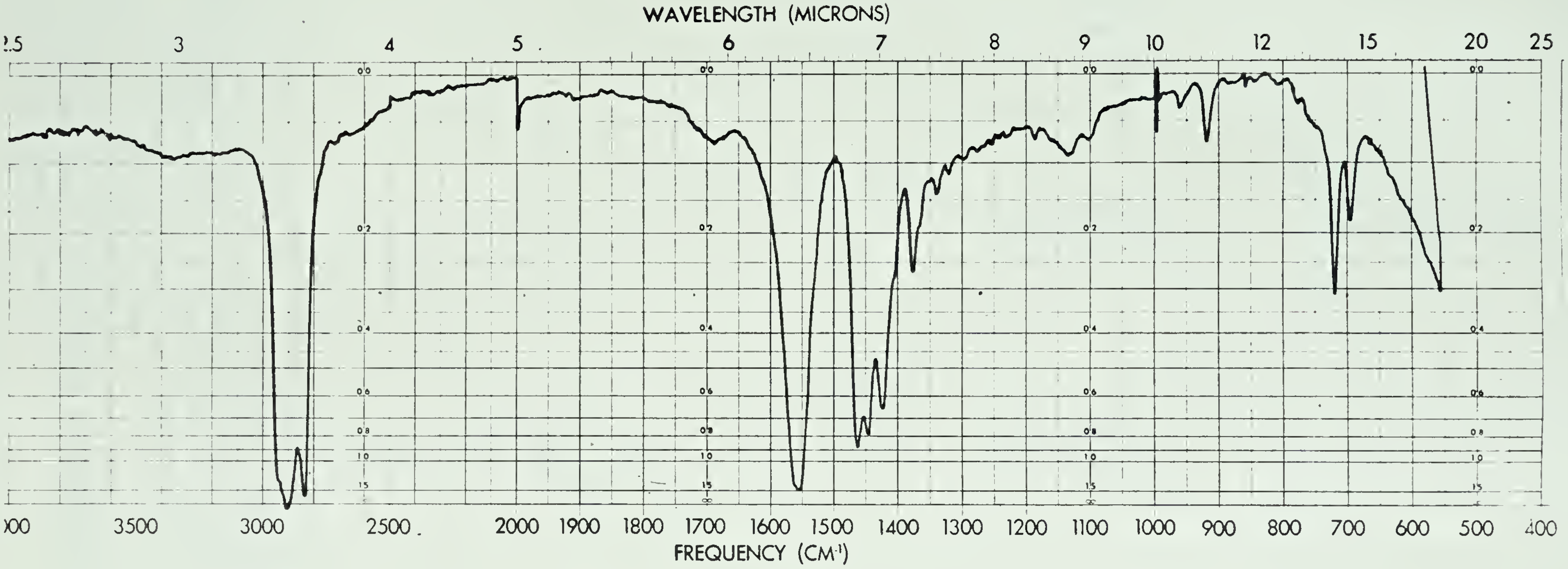
a. Underlined figures indicate that the 10% difference is in favour of the interesterified fat.

Table XIV. Infra-red absorpotion peaks and short spacings from X-ray diffraction obtained with milkfat and high melting fraction, either slowly or rapidly cooled and high melting fraction (HMF) crystallized from acetone.

Sample and Treatment	Wavenumber (cm^{-1})		Short Spacings Å			
milkfat slowly cooled	718(s)[a]	728(sh)[b]	4.64	4.17	3.78	
milkfat fast cooled	718(s)	727(sh)	4.57	4.11	3.83	
HMF slowly cooled	718(s)	727(sh)	4.23	4.13(w)[c]		3.84
HMF fast cooled	720(s)	727(wsh)[d]	4.11	3.85(w)		
HMF crystallized from acetone	717(s)		4.61	3.88	3.75	3.60(w)

a. strong peak
b. shoulder
c. weak
d. weak shoulder

Figure VII. Infra-red spectrum of residue remaining in the column after separation of mono, di and triglycerides.



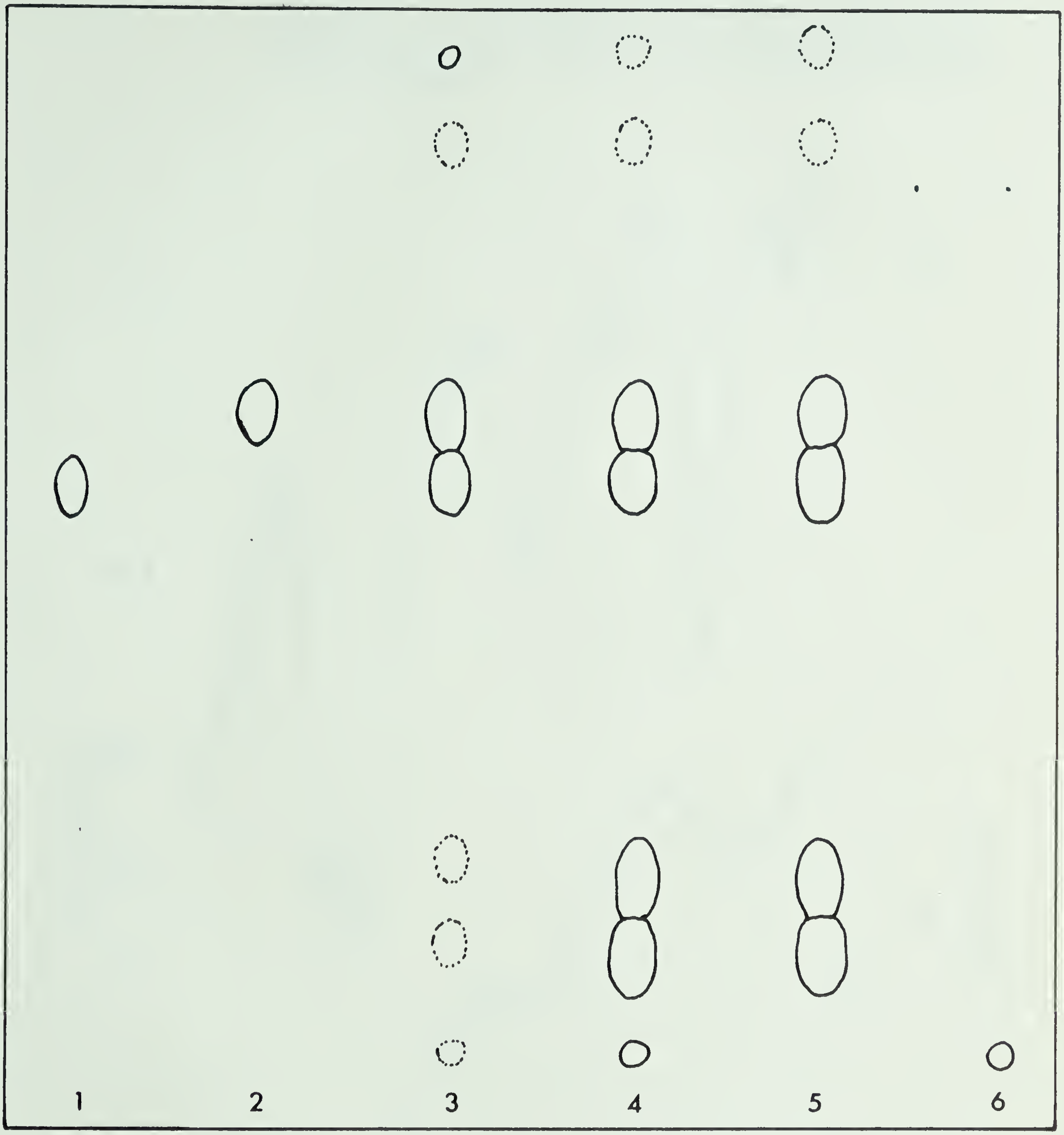


Figure VIII. Thin layer chromatogram of milkfat, hydrolyzed milkfat and lipid classes separated by column chromatography.
1. tributyrin 2. trimyristin 3. residue milkfat
4. hydrolyzed residue 5. first elution from column chromatograph 6. second elution.
The spots in ascending order are monoglycerides, 1:2 diglycerides, 1:3 diglycerides, short chain triglycerides, long chain triglycerides and impurities.

Figure IX. Infra-red spectra of the 2-monoglycerides obtained after pancreatic lipase hydrolysis of the -15°C fraction of milkfat. Curve A is the spectrum obtained with a film between NaCl plates, curve B is the spectrum obtained in a CS_2 solution.

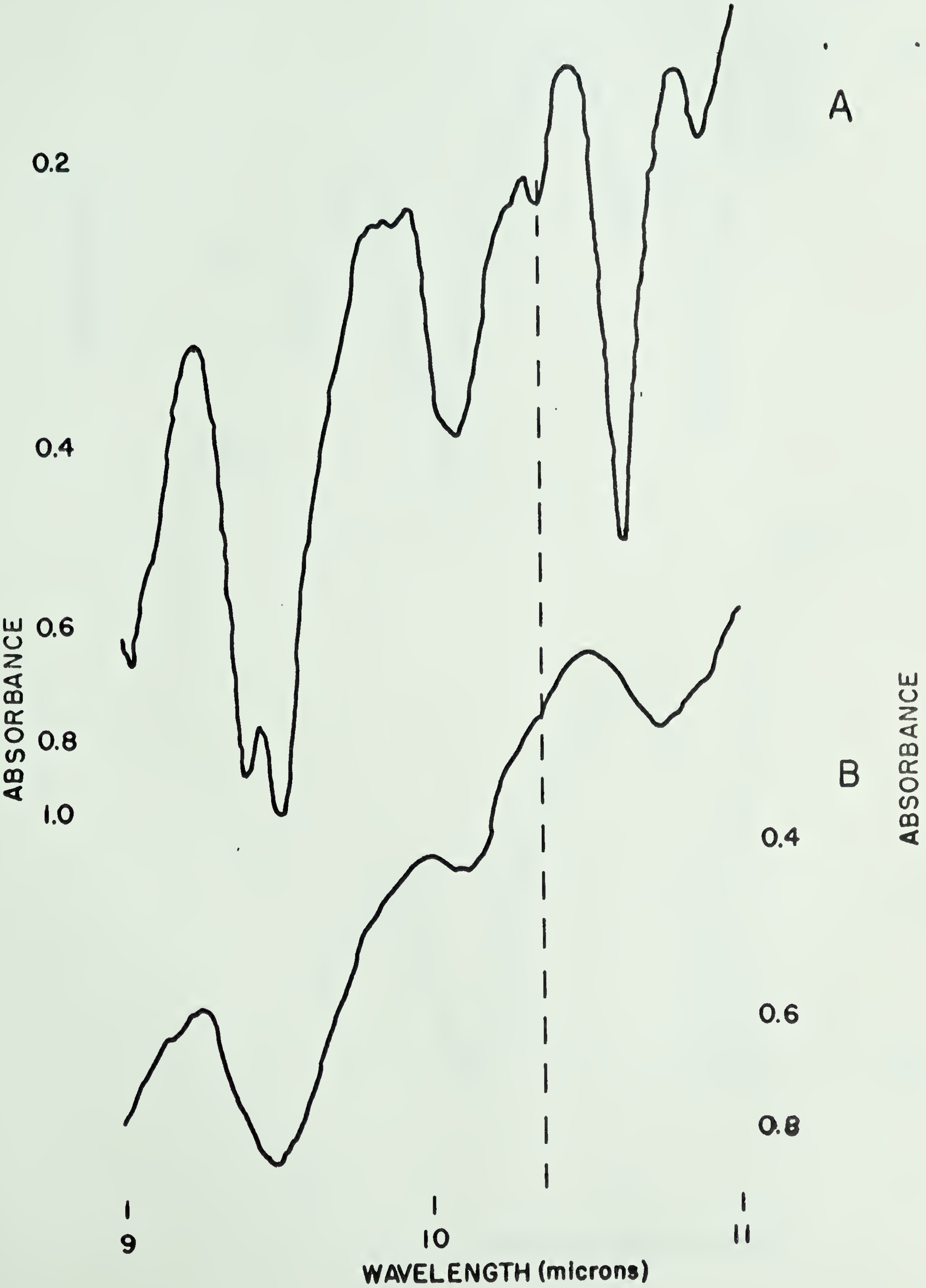


Figure X. Infra-red spectra of the 2-monoglycerides obtained after pancreatic lipase hydrolysis of trielaidin.

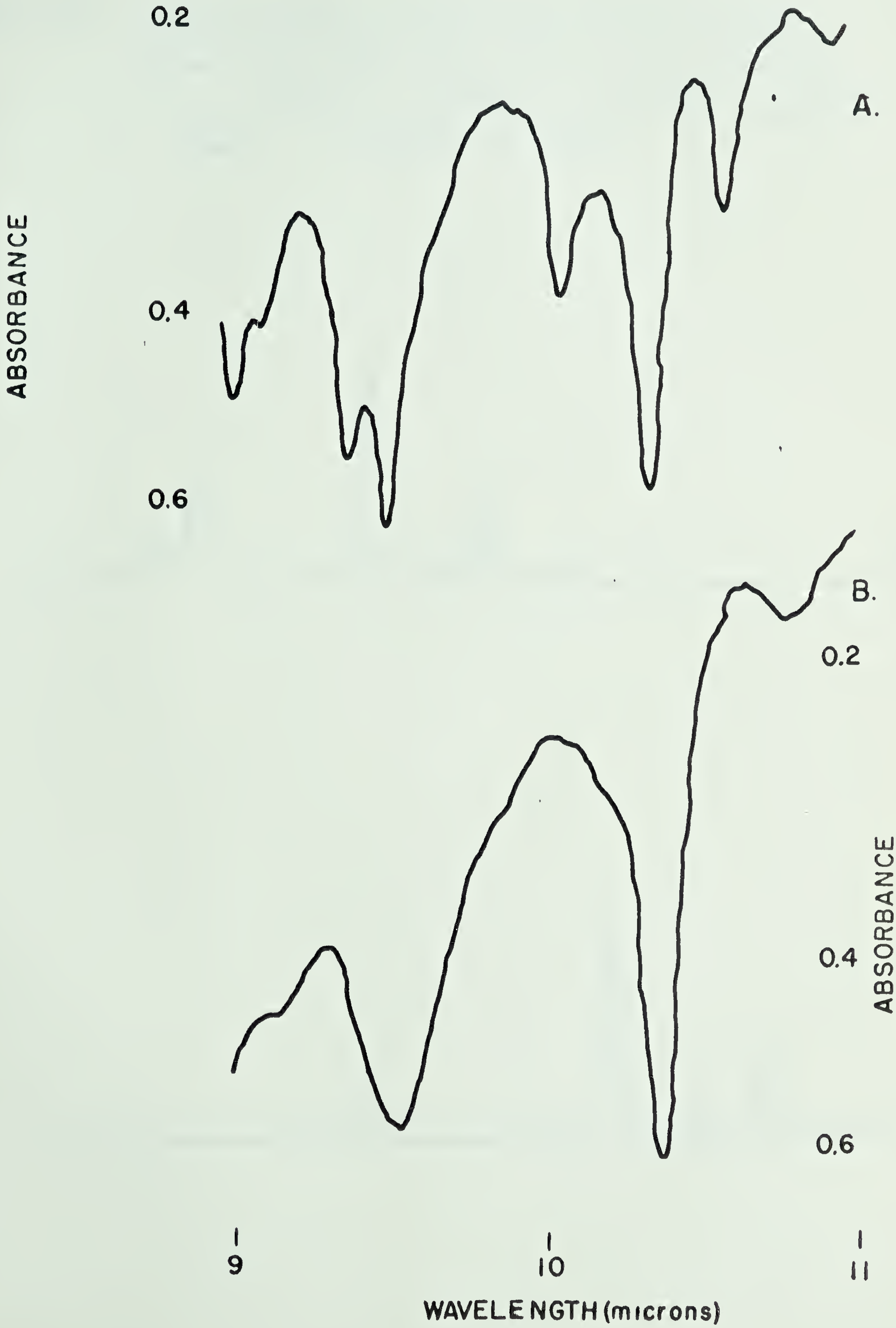


Figure XI. Gas chromatograms of methyl esters of +15°C milkfat fraction (A) and the methyl esters of the 2-monoglycerides of the +15°C milkfat fraction after hydrolysis (B). a. butyric, b. caproic, c. caprylic, d. capric, e. lauric, f. myristic, g. group A, h. palmitic, i. group B, j. stearic, k. oleic.

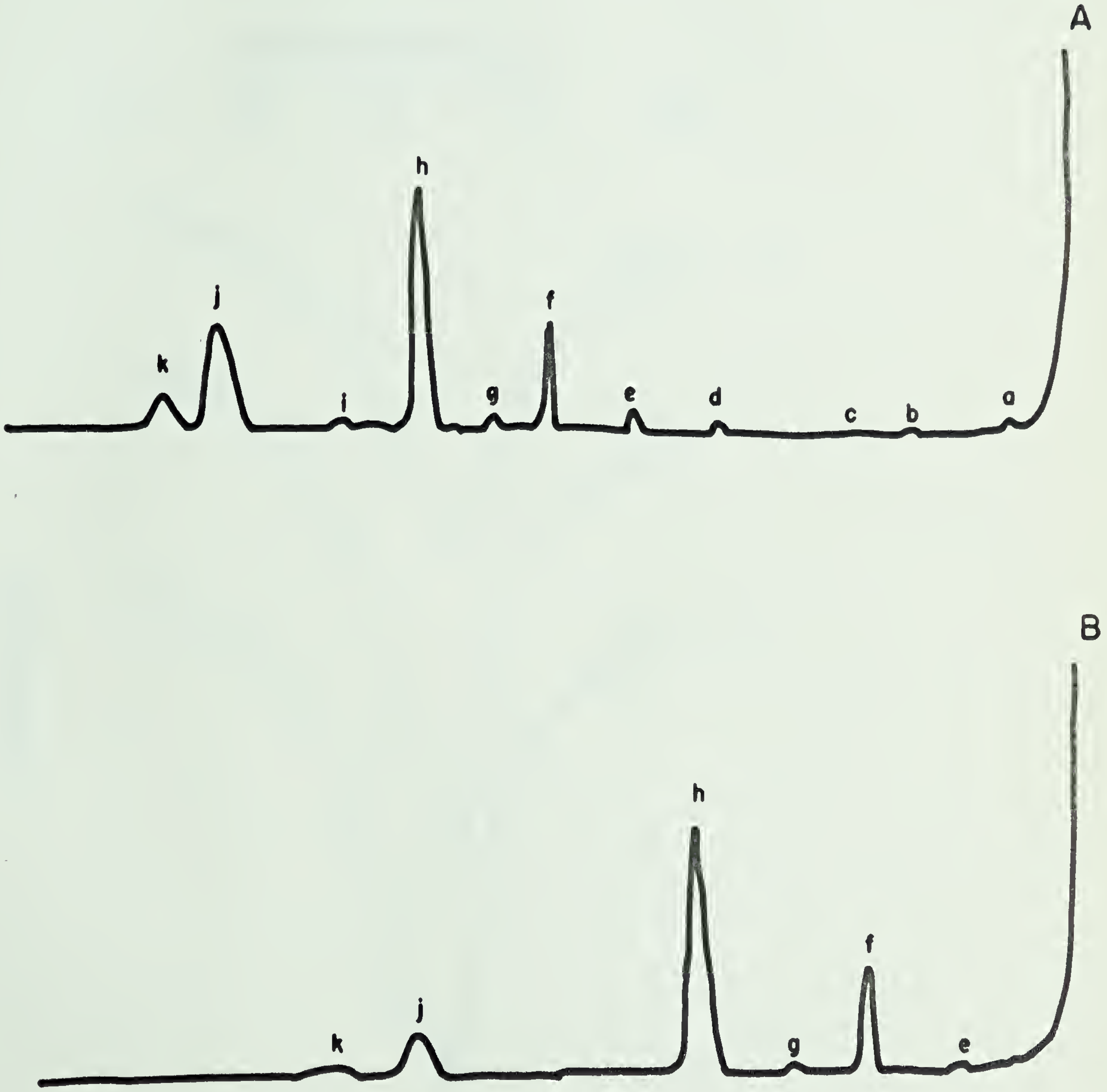


Figure XII. Infra-red spectra of milkfat at 40°C (A), after rapid cooling to 0°C (B), and with subsequent holding at 5°C for 10 hours (C).

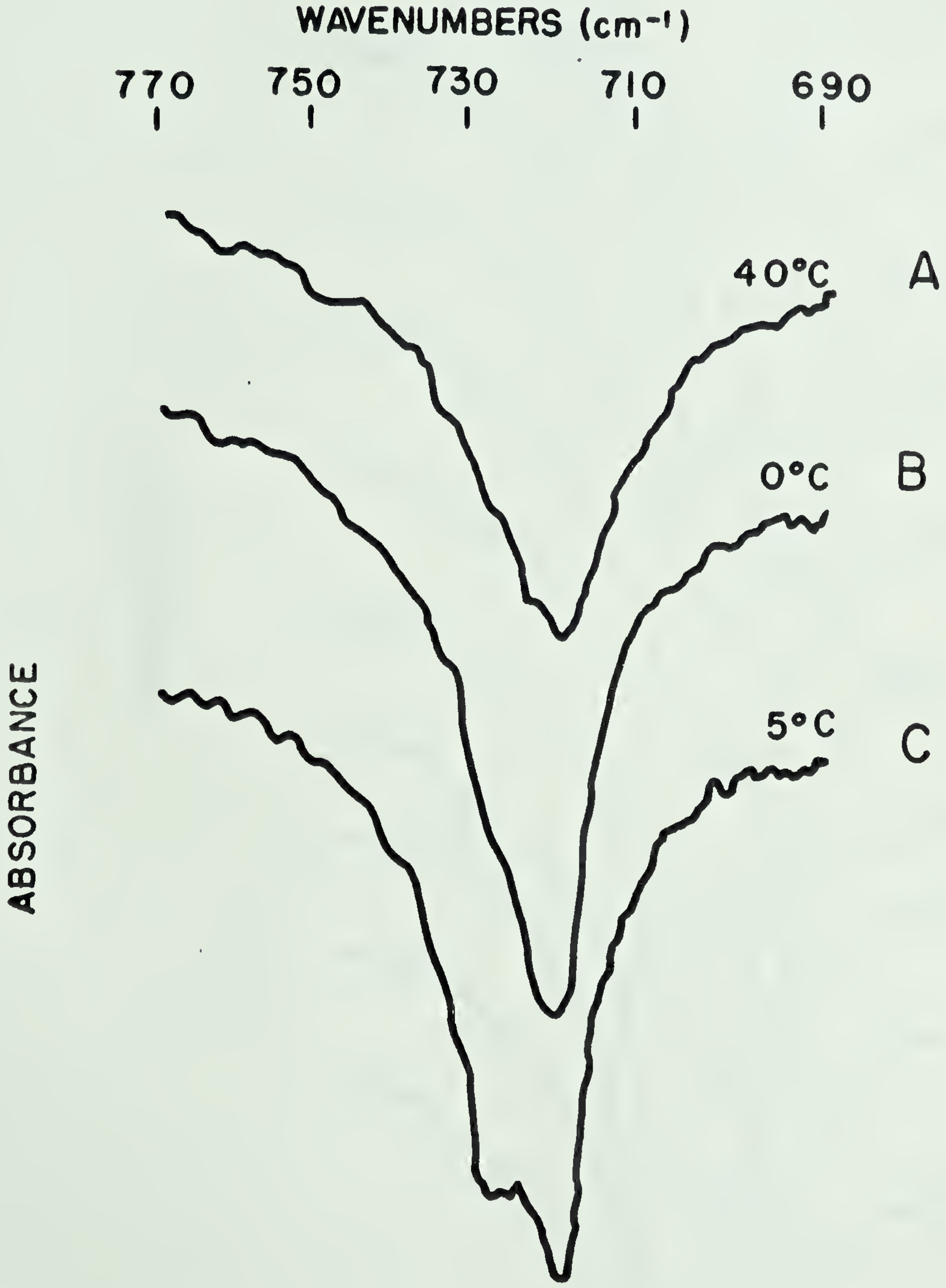


Figure XIII. Infra-red spectra of milkfat determined during cooling of the fat from 40-0°C at 4°C intervals.

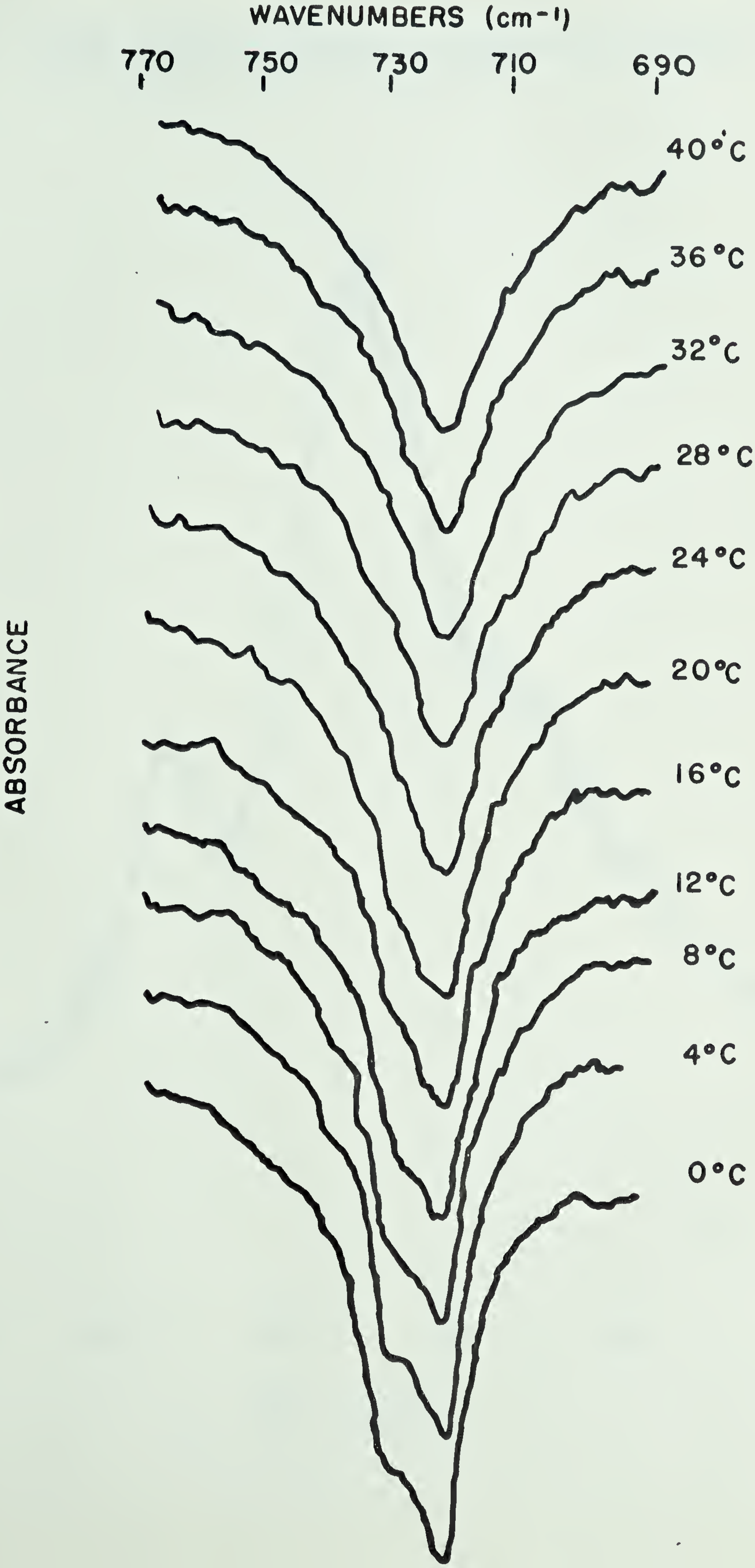


Figure XIV. X-ray diffraction pattern of slowly cooled milkfat after cooling slowly from 40-0°C.

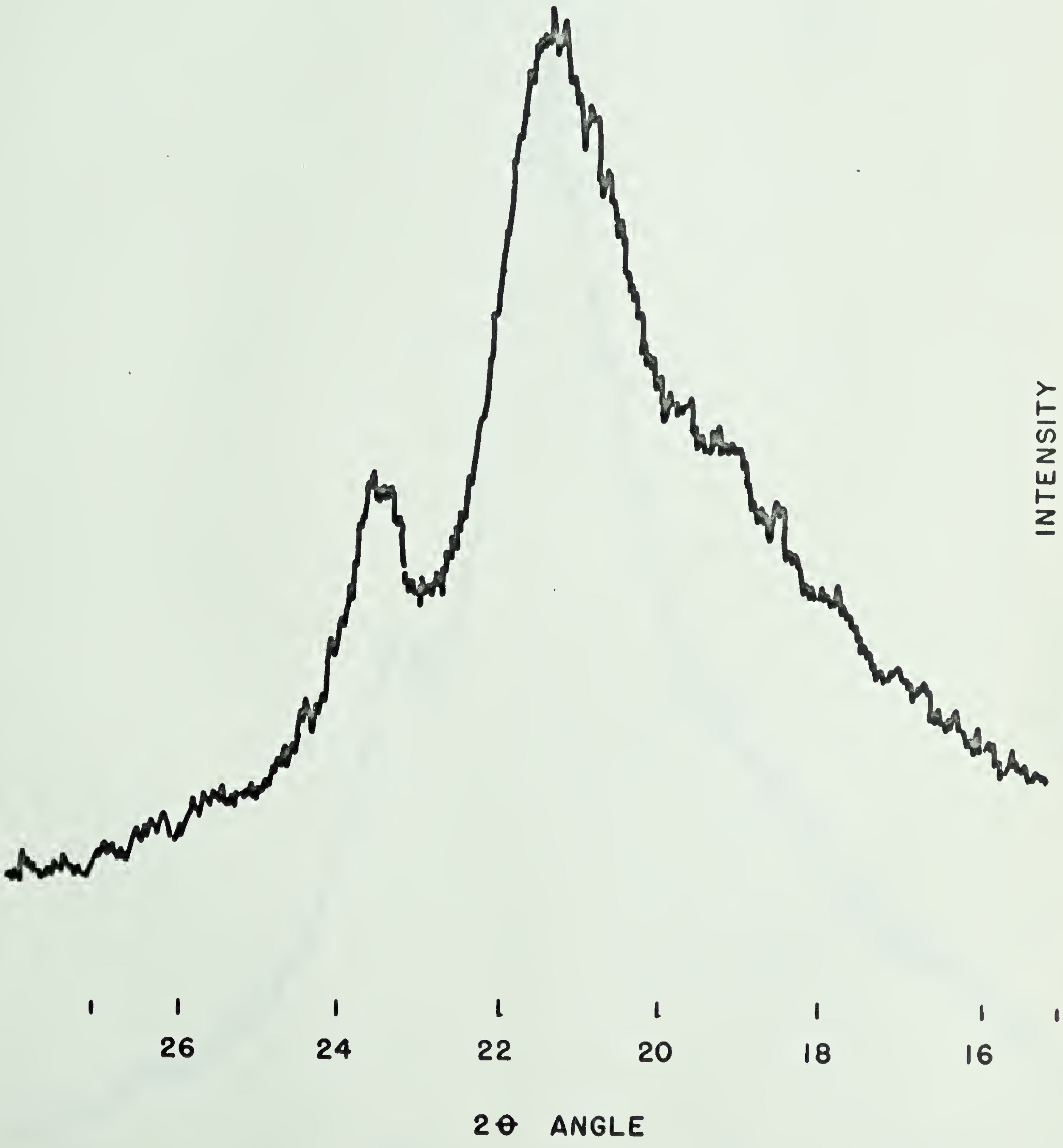


Figure XV. X-ray diffraction pattern of rapidly cooled milkfat after cooling rapidly from 40-0°C by dry ice.

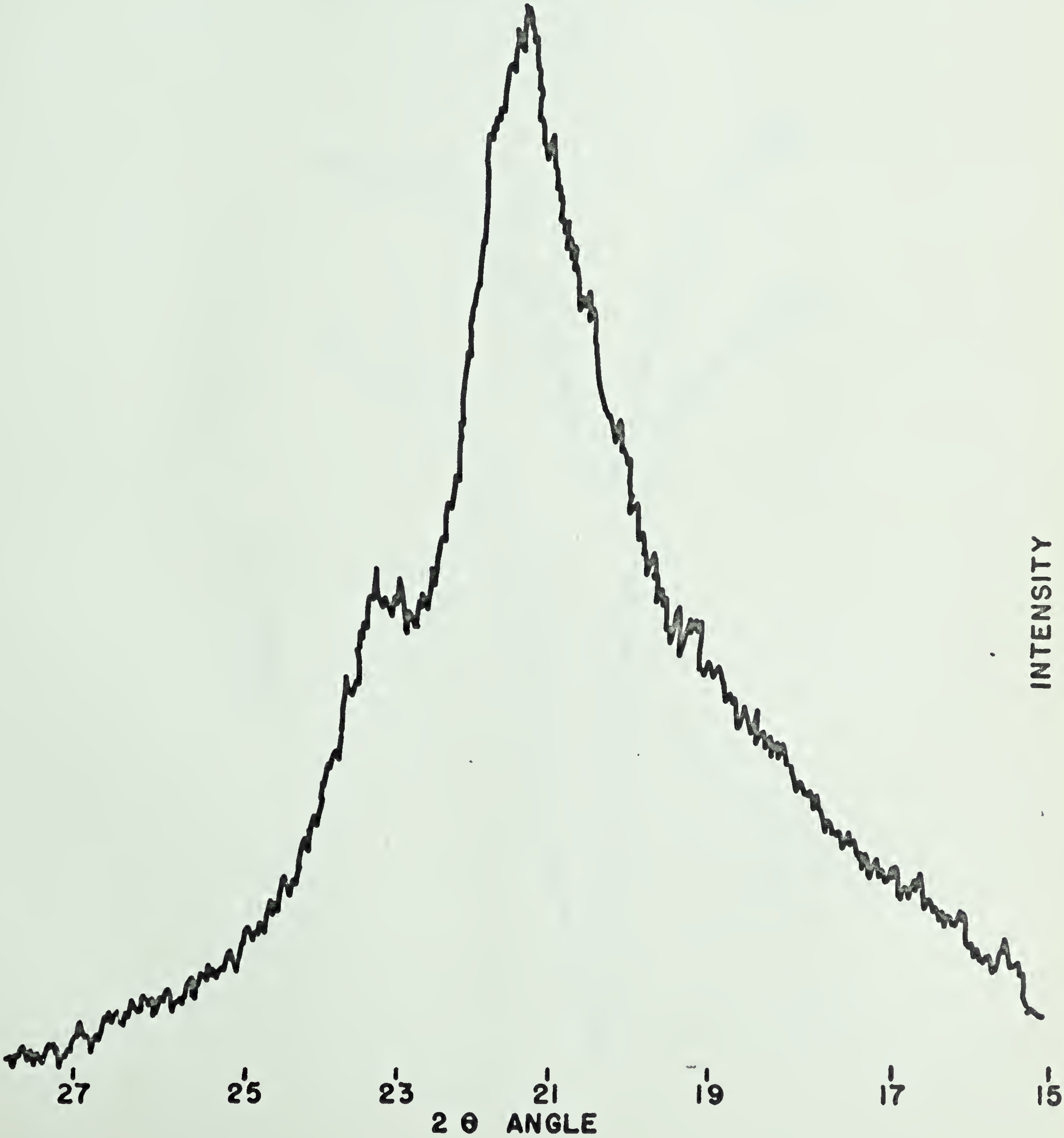


Figure XVI. Infra-red spectra of high melting glyceride fraction (+15°C acetone crystallization) at 65°C (A) and after rapid cooling to 10°C (B).

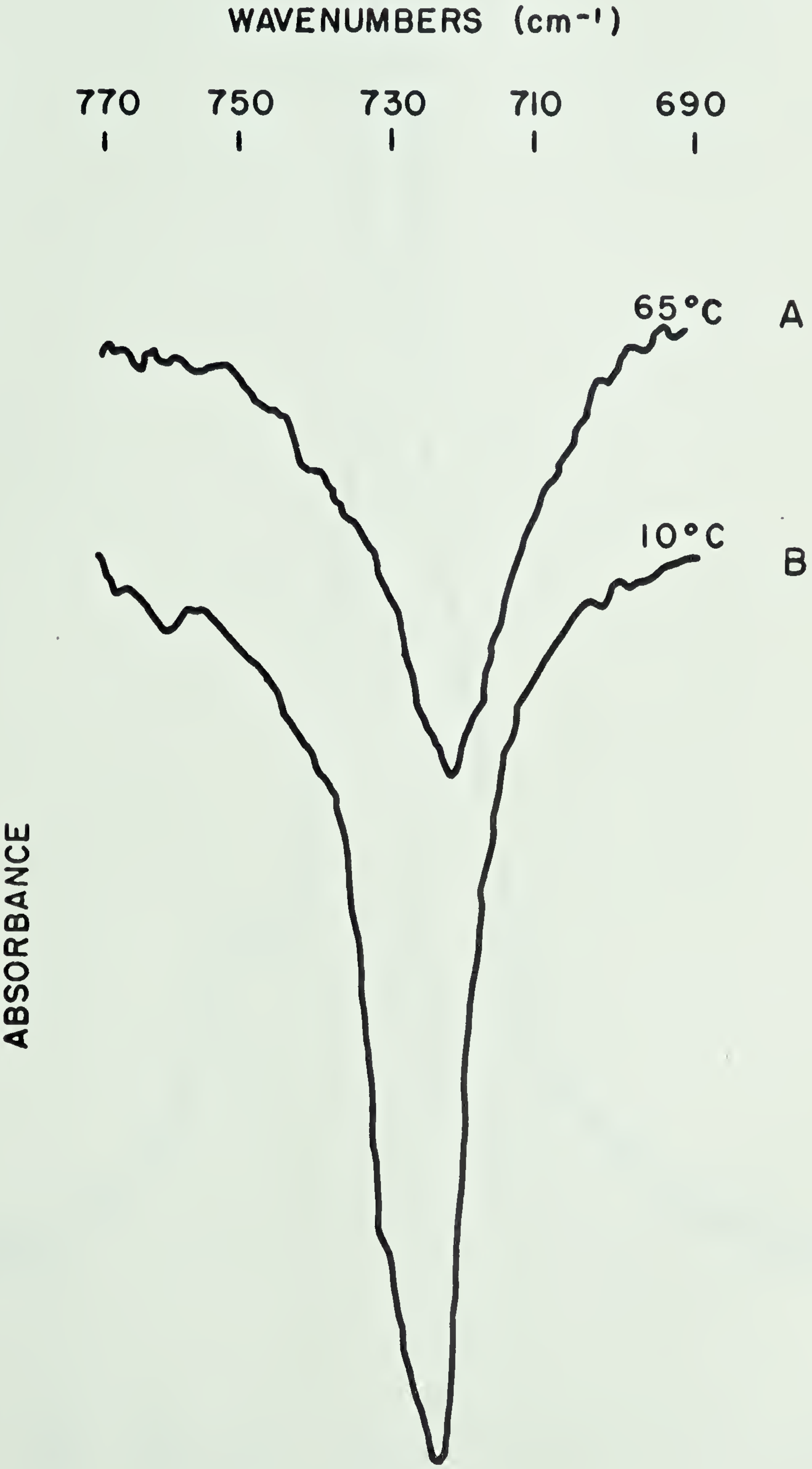


Figure XVII. X-ray diffraction pattern of high melting fraction cooled rapidly from 60°C to 10°C and run at room temperature.

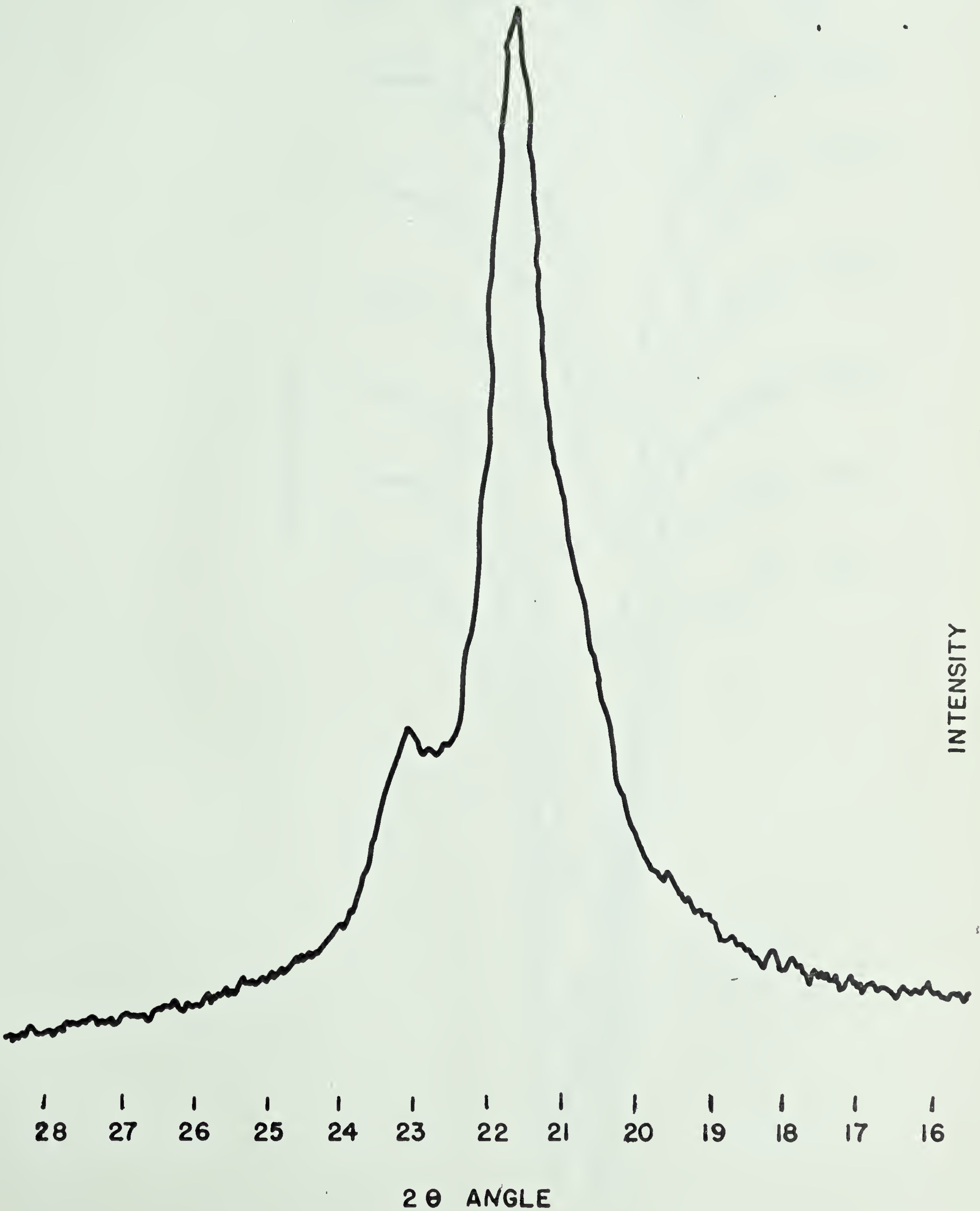


Figure XVIII. Infra-red spectra of high melting fraction cooled slowly from 60°C to 10°C at 4°C per hour.

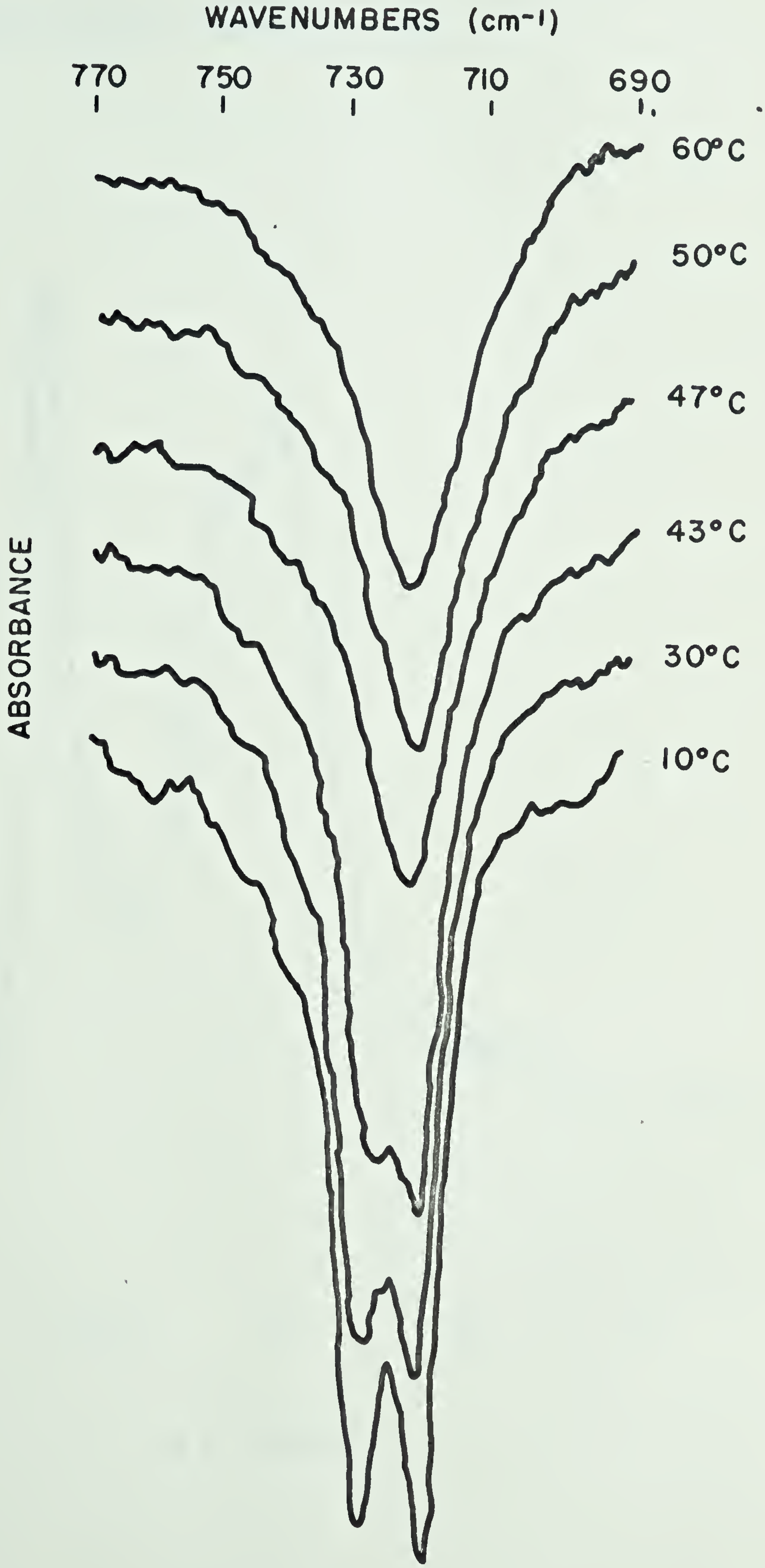


Figure XIX. X-ray diffraction pattern of slowly cooled high melting fraction from 65°C to 10°C.

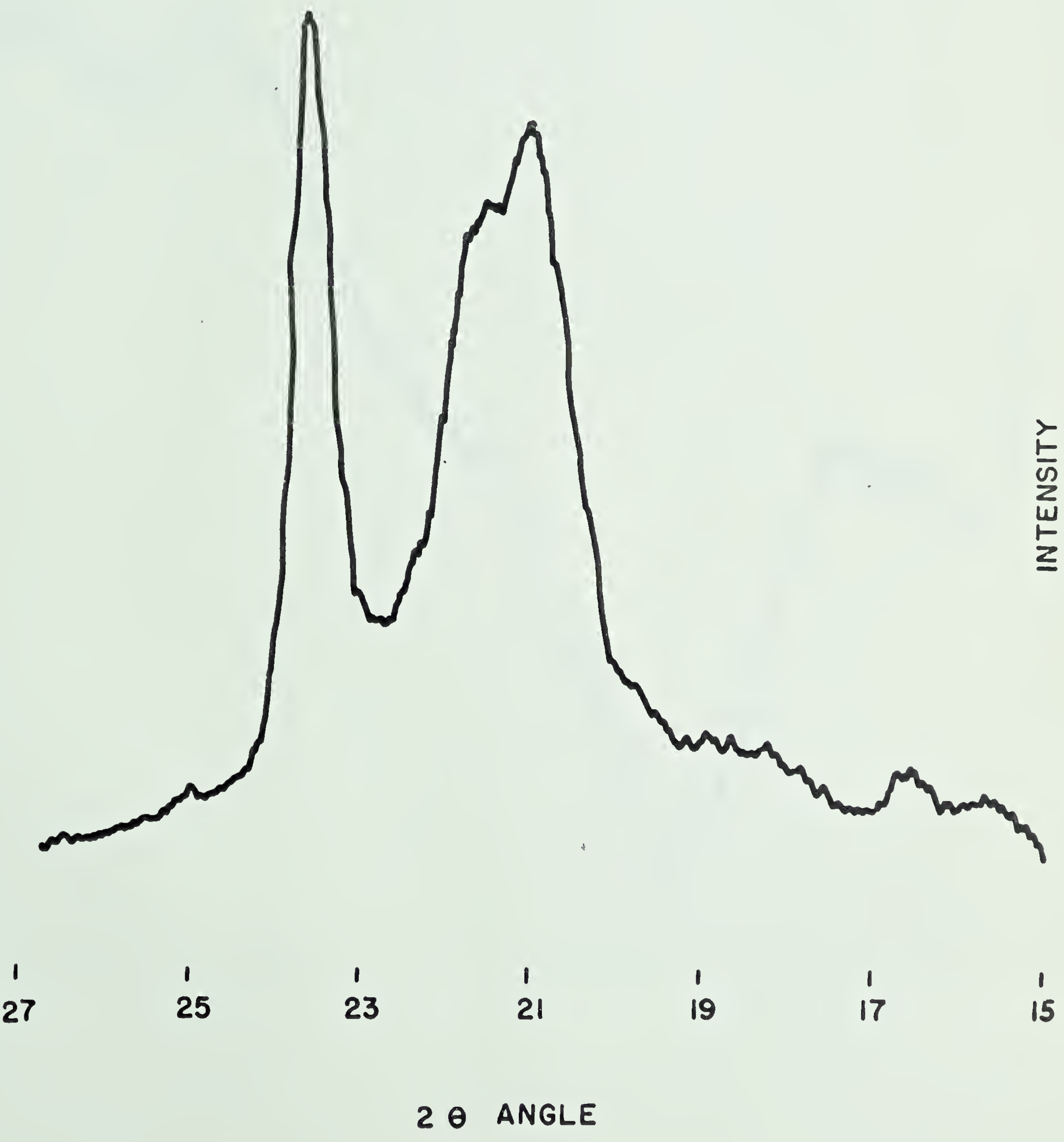


Figure XX. Infra-red spectrum of high melting fraction crystallized from acetone.

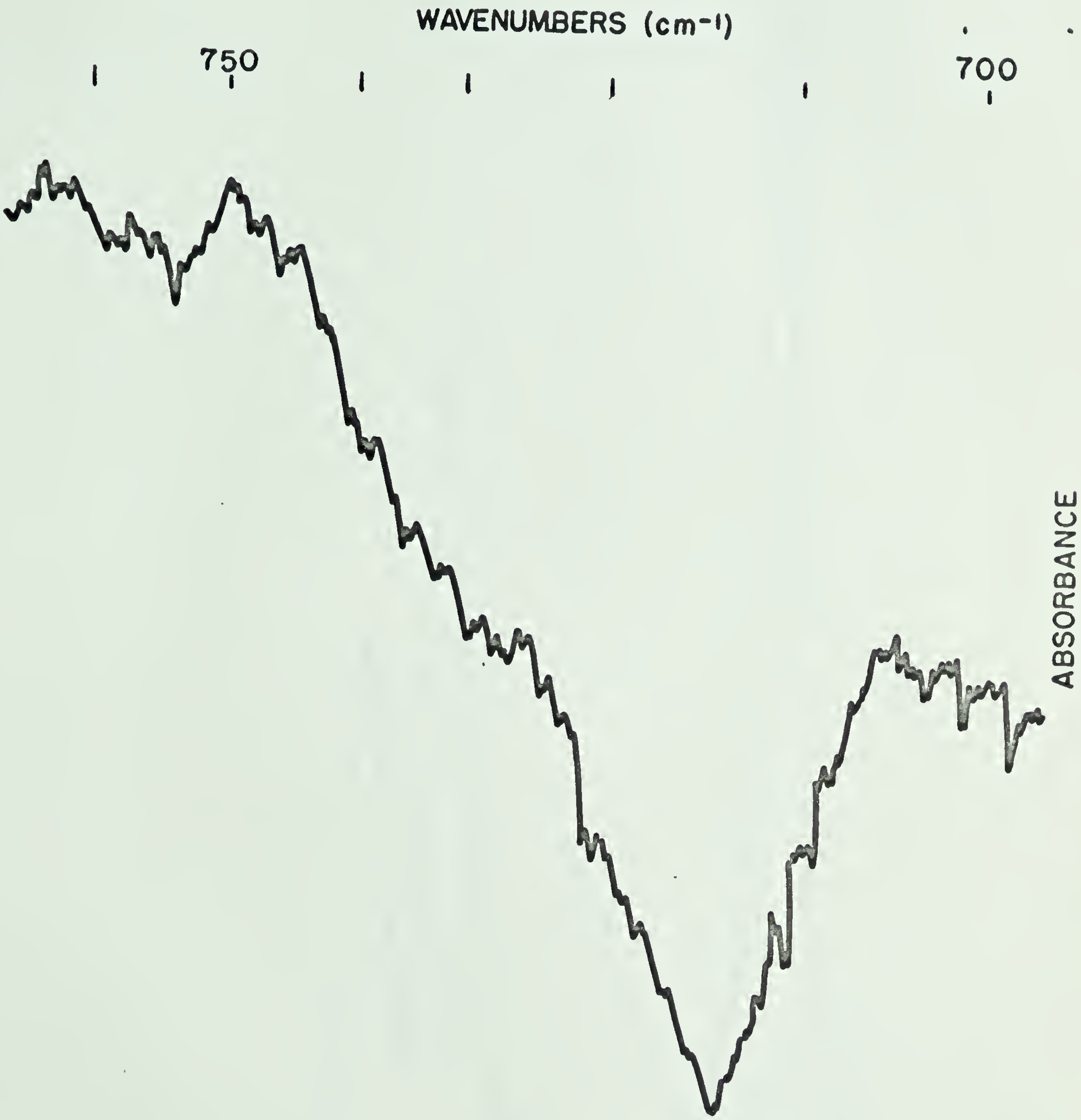


Figure XXI. X-ray diffraction pattern of high melting fraction crystallized from acetone.

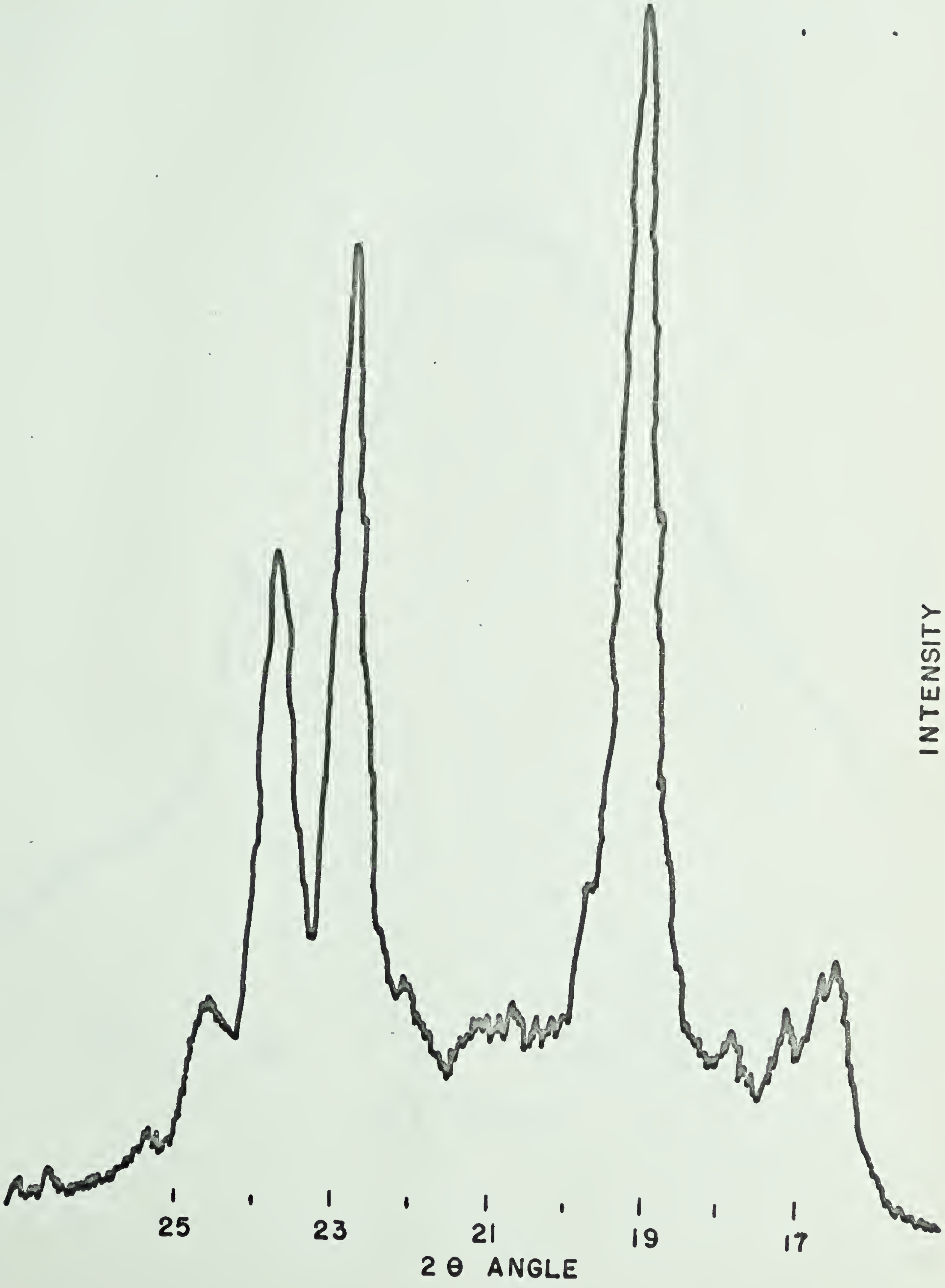


Figure XXII. X-ray diffraction pattern of milkfat crystallized at room temperature (25°C). Determination at 25°C.

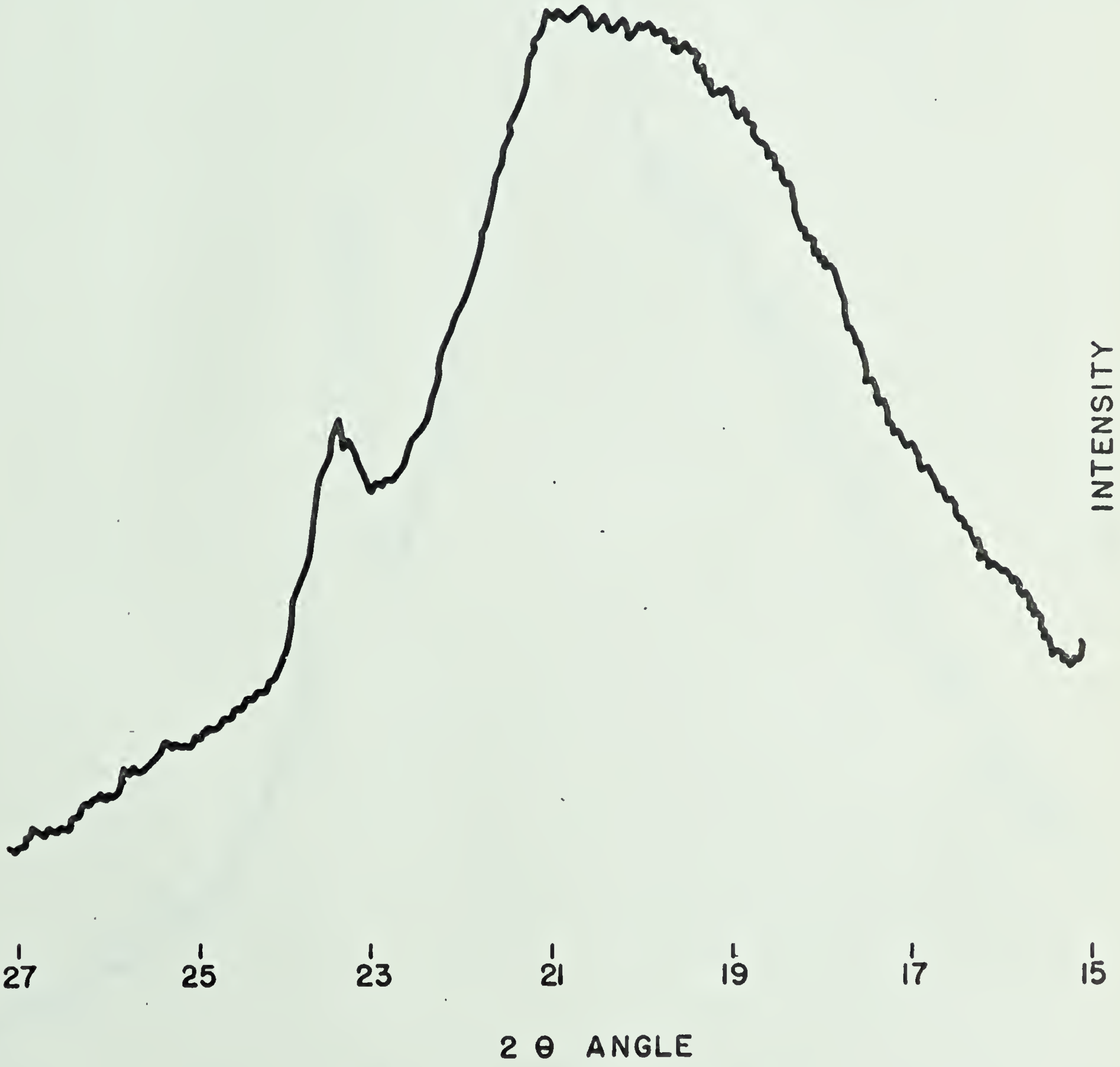
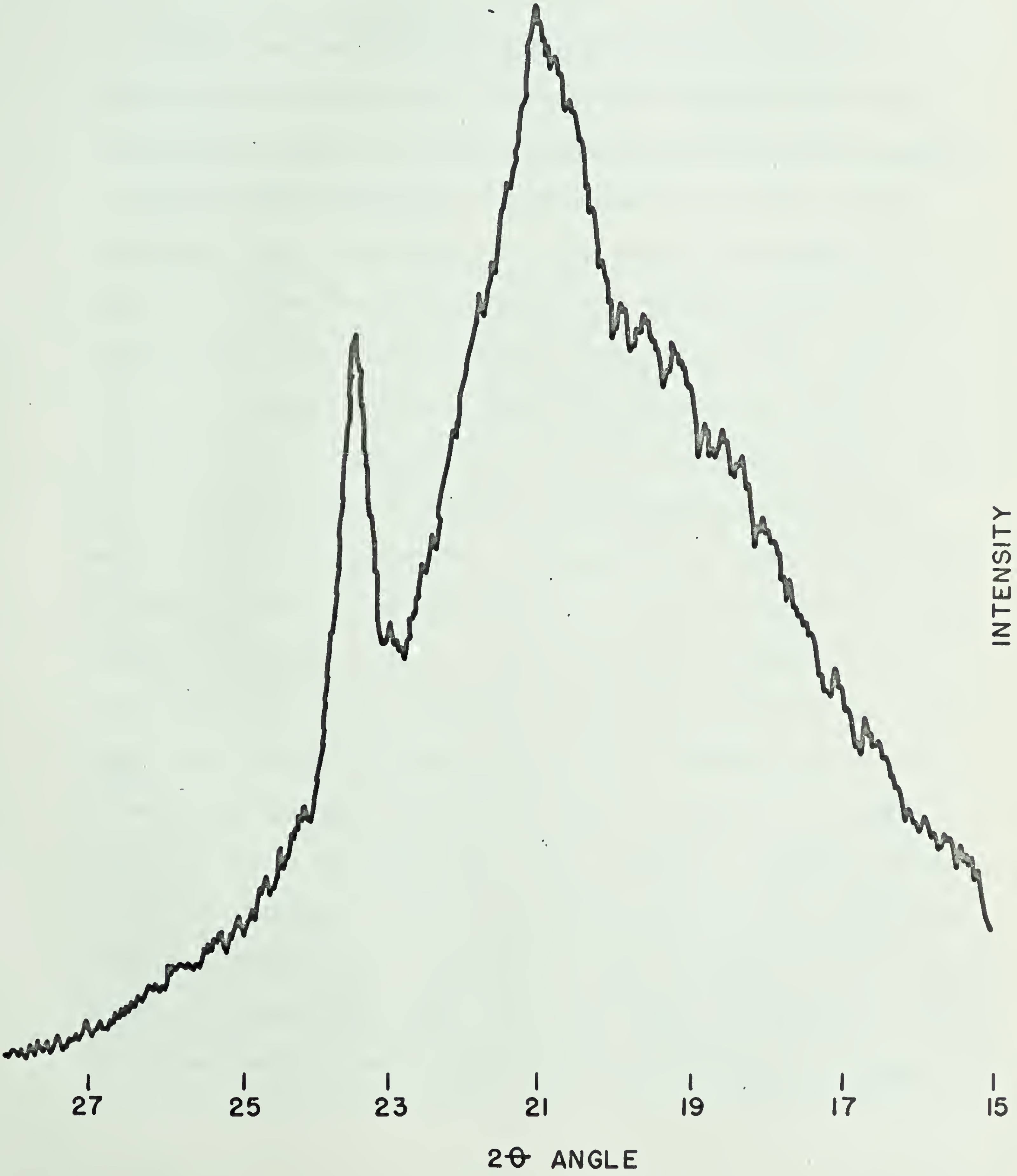


Figure XXIII. X-ray diffraction of the solid portion of milkfat crystallized at room temperature and separated from the liquid by pressure filtration. Determination at 25°C.



DISCUSSION

TRANS DISTRIBUTION IN MILKFAT

Milkfat was fractionated into eight fractions by fractional crystallization from acetone. This procedure segregates the milkfat into glyceride groups with melting points decreasing as the temperature of crystallization decreased. The saturated fatty acids, palmitic and stearic, were concentrated in the fractions which crystallize above 0°C, whereas the short chain and unsaturated fatty acids were concentrated in the fractions which crystallize below 0°C. Calculation of the trans content of these fractions gives an insight into the distribution of trans acids in the various glyceride groups of the fat. Interesterification results in randomization of fatty acids among and within the glycerides. The mechanism of interesterification has been suggested to involve a beta-keto ester as intermediate (Weiss, Jacobson & Wiedermann, 1961). The interesterified milkfat was also fractionated into eight fractions by fractional crystallization. The trans fatty acids have melting points that lie between those of the corresponding cis and saturated fatty acids. Because of the higher melting points of the trans fatty acids, it would be expected that the proportion of trans acids would be higher in the fractions obtained at the higher temperatures. The milkfat fraction obtained at 15°C contained 81.6% of the unsaturated fatty acids in the trans configuration, whereas in the residue this level was only 15.8%. The percentage of trans fatty

acids in all of the milkfat fractions was remarkably similar, about 9% (Table X). The interesterified milkfat fractions also contained approximately 9% trans unsaturation. The equal distribution of the trans fatty acids among the milkfat fractions obtained by fractional crystallization from acetone and similarly in the interesterified fractions shows that the trans fatty acids are distributed in a random fashion among the glyceride groups.

The distribution of trans fatty acids within the glycerides of milkfat and its fractions was determined by subjecting the samples to pancreatic lipase hydrolysis. The 2-monoglycerides isolated from the hydrolysis mixture of milkfat and its fractions contained only trace amounts of trans fatty acids. If the level of any fatty acid present in the 2-monoglycerides does not exceed 33.3%, it is said that this fatty acid is preferentially esterified in the 1 and 3 positions of the glycerides. Thus, it can be concluded that the trans fatty acids in milkfat are esterified preferentially in the 1 and 3 positions. After interesterification the fatty acids should be rearranged in a random fashion among and within the glycerides. The completeness of randomization can be determined by pancreatic lipase hydrolysis and determination of the percentage of the fatty acids in the 2-position. Theoretically, after complete randomization all of the fatty acids should be present at the 2-position at a level of 33.3%. In the interesterified fat the percentage of individual fatty acids at the 2-position was found to range between 25-40%. Only traces of trans fatty acids

were found in the 2-position after hydrolysis of the interesterified milkfat and its fractions.

Jensen, Sampugna & Pereira (1964) showed that pancreatic lipase did not appear to differentiate to any extent between elaidic acid and its cis isomer oleic acid, or linoleic and lauric acids. To establish that trans 2-monoglycerides exhibit a trans peak at 10.36μ, a sample of trielaidin was subjected to pancreatic lipase hydrolysis. The resulting monoglycerides did behave in the normal fashion for trans unsaturation. The percentage trans as trielaidin was found to be 68%. To convert this figure to percentage trans as monoelaidin, this percentage must be multiplied by a factor obtained by dividing the molecular weight of monoelaidin times three by the molecular weight of trielaidin. The percentage trans as monoelaidin was calculated to be 82.1%. As the trans content of the trielaidin was found to be 88.1% this was an indication that the procedures used do not produce artifacts.

It appears, therefore, that trans unsaturated fatty acids are not normally present at the 2-position of milkfat glycerides and will not be moved there by the randomization process. This suggests that trans fatty acids are incorporated into glycerides at the 2-position with much greater difficulty than cis fatty acids, because oleic acid was found to be randomly distributed after interesterification. Some recent work by Raulin, Loriette & Clement (1963) seems to support this conclusion. They fed peanut oil enriched with elaidic acid to the rats; by using pancreatic lipase and infra-red spectroscopy, they

determined that the trans fatty acids were incorporated preferentially in the 1 and 3 positions of the glycerides in depot fat, epididymal pads and body fat of the rats. It seems possible that the non-incorporation of trans unsaturated fatty acids in the 2-position will be a general principle in the structure of natural fats.

FATTY ACID DISTRIBUTION IN MILKFAT

The proportions of fatty acids in the 2-position (Table Xl) shows that some fatty acids are preferentially esterified in the 2-positions of milkfat glycerides. A value of 33.3% of a fatty acid in the 2-position indicates that the fatty acid is not preferentially esterified in any position of the glycerides. Smith, Freeman & Jack (1965) have pointed out that a possible error of 5% in the determination of the percentage fatty acid in the 2-position is reasonable. Thus, if the percentage of the fatty acid in the 2-position exceeds 38.3%, the fatty acid can be said to be preferentially incorporated in the 2-position, whereas if the percentage is less than 28.3%, the fatty acid is preferentially esterified in the 1 and 3 positions. The results show that there is preferential incorporation of lauric, myristic, group A fatty acids, and palmitic acid in the 2-position of all milkfat fractions. Stearic and oleic acids are preferentially esterified in the 1 and 3 positions. These results agree with some published data, (Smith, Freeman & Jack, 1965; Blank & Privett, 1964 and Boudreau & deMan, 1965).

CHARACTERIZATION OF RESIDUE REMAINING IN COLUMN AFTER ELUTION OF PARTIAL GLYCERIDES

During hydrolysis by pancreatic lipase the resulting fatty acids are continuously neutralized by alkali and the resulting reaction mixture, therefore, contains fatty acid salts in addition to triglycerides and partial glycerides. Stinson (1966) found that it was important that the ether solution be washed completely free of pancreatic lipase and bile salts in order to obtain satisfactory separation of lipid classes by column chromatography. At least a portion of the sodium salts of the carboxylic acids should have been removed by the washing procedure, but another portion may be applied to the chromatographic column. The separation of lipid classes by Florisil column chromatography yielded a recovery of 65% of lipid material. The remaining material in the column was eluted with 100 ml of methanol. The methanol was evaporated and an infra-red spectrum was obtained of the residue (Figure VII).

This residue was thought to be sodium salts of long chain carboxylic acids. The spectrum obtained was compared to those obtained by Chapman (1958b) who examined anhydrous sodium soaps. All anhydrous sodium soaps examined exhibited bands near 1560 cm^{-1}, 1415 cm^{-1} and at 695 cm^{-1} which result from C-O stretching vibrations and CO.O deformation. Also observed in all the spectra were two absorption bands at 1460 cm^{-1} arising from CH_2 symmetrical deformation mode and 1380 cm^{-1} from CH_3 symmetrical bending mode. The band at 1440 cm^{-1} was assigned

to the vibration of the CH_2 group adjacent to the carboxylate group. CH_2 wagging modes gave rise to absorption band in the 1200-1350 cm^{-1} region. The band at 720 cm^{-1} was assigned to the CH_2 rocking vibration. From the appearance of these absorption bands in the spectrum of the residue, it was concluded that the residue represented sodium salts of carboxylic acids. These are most likely to be long chain fatty acids because of the nine bands in the region of 1200-1350 cm^{-1} which are used qualitatively to determine the chain length of the sodium soaps (Brown & Sheppard, 1954).

POLYMORPHISM

The occurrence of polymorphism in milkfat was first suggested by Mulder (1940) who observed a double melting point. Thomas (1950) and Greenbank (1952) confirmed the existence of polymorphism in milkfat by observing the presence of two polymorphic forms which were designated beta and beta-prime. Four polymorphic modifications were observed by Tverdokhleb (1962) and Beloussov & Vergelessov (1962), they were named gamma or glassy, alpha, beta-prime and beta. It was mentioned before that there is now considerable doubt about the existence of a gamma or glassy form (Chapman, 1962).

X-ray diffraction analysis and infra-red spectroscopy were used to establish the presence of the different polymorphic forms in milkfat and in its high melting fraction (HMF). The results of the diffraction patterns and spectra were interpreted with the use of literature data (Table XV). The infra-red spectrum of liquid fat exhibits a broad peak

Table XV. Polymorphic literature data from infra-red spectra and short spacings from X-ray diffraction.

Polymorphic Form	Wavenumber(cm^{-1})	Short Spacings Å	
	Chapman (1962)	Lutton (1950)	Hannowijk (1964)
alpha	720	4.14	4.14
beta-prime	727 719	3.87 4.18	3.78 4.18
beta	717	3.68 3.84 4.68	3.68 3.86 4.59

at 720 cm^{-1}. Upon rapid cooling from 40°C to 0°C the absorbance increased which indicates the formation of the alpha polymorph. The temperature could not be kept at 0°C because of fogging of the windows in the Variable Temperature Chamber. Therefore, the milkfat was allowed to remain at 5°C for 10 hours. The infra-red spectrum run after this holding time, exhibited a shoulder at 727 cm^{-1}, and a strong absorption band at 718 cm^{-1}. This indicates that the milkfat existed in more than one polymorphic form including the beta-prime and beta forms. The broadness of the peak at 720 cm^{-1} is due to the presence of liquid oil. The X-ray diffraction pattern of the rapidly cooled milkfat showed that the crystalline milkfat had short spacings of 4.57Å, 4.11Å and 3.83Å. The short spacing of 4.11Å indicated the presence of the alpha polymorph, the spacing of 3.83Å the beta-prime and the spacing of 4.57Å the beta form. The X-ray diffraction pattern of slowly cooled milkfat indicated the simultaneous presence of the three polymorphic forms.

Polymorphism of complex glyceride mixtures such as milkfat with a melting range of 40°C to -40°C has no law or order of prediction of the polymorphic forms that may occur under certain conditions. Rapid cooling of milkfat leads to the formation of mixed crystals. These crystals may occur in different polymorphic modifications, mainly the alpha form, this was shown by the infra-red spectrum. After tempering at 5°C for 10 hours rearrangement of the mixed crystals may occur, and also a transformation of the alpha polymorph to the beta-prime polymorph shown by the infra-red spectrum. The X-ray diffraction pattern

also indicated the presence of the beta form. Slow cooling of milkfat resulted in less mixed crystal formation, and all three polymorphic forms were found to be present.

The HMF of milkfat consists of glycerides with melting range of 15-60°C. Therefore, if the cooling goes below 15°C the HMF is totally solid, so that no liquid oil is present to interfere with the formation of certain polymorphic modifications. The HMF crystallized from acetone was shown to exist in the beta modification. The short spacings were found to be 4.61Å, 3.88Å and 3.60Å. The infra-red spectrum indicated an absorption maximum at 717 cm^{-1}. Rapid cooling of the HMF from 65°C to 10°C produce a crystalline solid, the X-ray diffraction pattern exhibited a short spacing of 4.11Å and the infra-red spectrum had an absorption maximum at 720 cm^{-1}. This indicates the presence of the alpha polymorphic form. Slow cooling of the HMF produced the beta-prime polymorph, which exhibits a doublet at 727 cm^{-1} and 718 cm^{-1} in the infra-red spectrum, and short spacings of 4.23Å and 3.84Å, calculated from the X-ray diffraction pattern.

In the determination of polymorphic modifications in fats the resolution of the infra-red spectra and X-ray diffraction patterns is affected by the presence of liquid oil. The oil or melt exhibits a broad absorption band at 720 cm^{-1} and the diffraction pattern in the region of 2θ angles of 15-30° is broad and diffuse as a result of scattering. An experiment was carried out to show that by removal of most of the oil from milkfat a much better diffraction pattern would be

obtained. An X-ray diffraction pattern was first obtained with milkfat which was kept at room temperature for 24 hours. The milkfat was subjected to pressure filtration and the crystalline portion was used to obtain the X-ray diffraction pattern. The resolution of the X-ray diffraction pattern compared to that of whole milkfat was considerably improved. It can be concluded from these results that if polymorphic forms were to be determined in a fat which consists of a liquid portion, the oil should first be removed before an X-ray diffraction pattern or infra-red spectrum is obtained.

From the study of polymorphism in milkfat, the occurrence of all the polymorphic forms in slowly and rapidly cooled milkfat indicate that the differences in physical properties of rapidly and slowly cooled milkfat were not likely to be caused by polymorphic changes.

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